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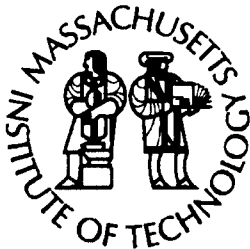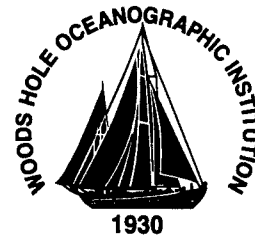
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Trace Metals and the Ecology of Marine Cyanobacteria

by

Elizabeth Lowell Mann

February 2000

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February 2000

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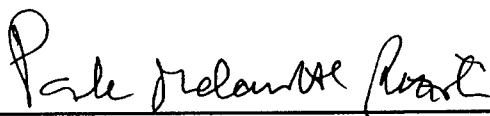
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TRACE METALS AND THE ECOLOGY OF MARINE CYANOBACTERIA

by

Elizabeth Lowell Mann

B.A., Bowdoin College, 1989

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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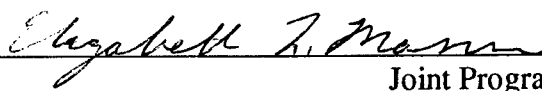
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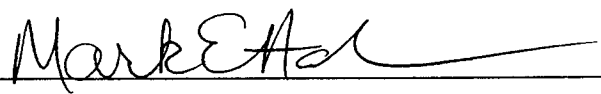
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TRACE METALS AND THE ECOLOGY OF MARINE CYANOBACTERIA

by

Elizabeth Lowell Mann

Submitted to the Department of Biology in February 2000 in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

ABSTRACT

The marine cyanobacteria *Synechococcus* and *Prochlorococcus* are important primary producers in oligotrophic oceans. The abundance and cell division rates of these cyanobacteria can be influenced by trace metals such as iron and copper. Iron is an essential trace metal that is present in the high nutrient, low chlorophyll waters of the equatorial Pacific in extremely low concentrations. When these waters were enriched with iron, *Prochlorococcus* chlorophyll fluorescence per cell and cell size increased. Cell division rates doubled inside the iron enriched patch and reached two divisions per day in bottle incubations with additional iron, indicating that *Prochlorococcus* were iron limited. However, cell numbers remained constant because mortality rates nearly doubled after the addition of iron and essentially matched the increases in cell division rate.

Trace metals can also be present in toxic, rather than limiting concentrations. Copper is an essential trace element that is toxic to cyanobacteria in pM quantities. In stratified water columns in the Sargasso Sea, free Cu^{2+} concentrations are high in the mixed layer (up to 6pM) and most of the *Prochlorococcus* population is located below the thermocline where free Cu^{2+} concentrations are lower. The distribution of *Synechococcus* is more uniform with depth. *Prochlorococcus* isolates were more sensitive to copper than *Synechococcus*, but members of the low chl B/A (high light adapted) ecotype were less sensitive than strains with high chl B/A ratios (low light adapted). In the field, the *in situ* concentration of free Cu^{2+} had a strong effect on the copper sensitivity of *Prochlorococcus*. Net growth rates were substantially reduced when *Prochlorococcus* from environments where the *in situ* free Cu^{2+} was low (deep mixed layers and below the thermocline in stratified water) were exposed to copper.

Prochlorococcus in shallow mixed layers where *in situ* Cu^{2+} was high were less sensitive to copper and may have been members of the copper resistant low chl B/A ecotype. *Synechococcus* were relatively copper resistant across a range of environments. These data are consistent with the hypothesis that ambient copper levels may influence the relative abundance of *Prochlorococcus* and *Synechococcus* in the Sargasso Sea.

Thesis Supervisor: Sallie W. Chisholm
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                                       Massachusetts Institute of Technology

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Chapter 1

Introduction

The marine cyanobacteria *Prochlorococcus* and *Synechococcus* are of interest for

several reasons. *Synechococcus* and *Prochlorococcus* are important primary

producers in oligotrophic oceans. *Prochlorococcus* alone can account for up to 50%

of the total chlorophyll *a* in oligotrophic regions (Velduis and Kraay 1993, Goericke

and Welschmeyer 1993, and Anderson et al. 1996) and represents 25% of the

seasonally averaged primary production in the Sargasso Sea (Goericke and

Welschmeyer 1993). Using flow cytometry, *Prochlorococcus* and *Synechococcus*

can be enumerated and distinguished from each other based on their pigment

composition. Flow cytometry can also determine the cell size and chlorophyll

fluorescence of *Prochlorococcus* and *Synechococcus* in the field. In addition, cell

division in *Prochlorococcus* is tightly synchronized to the light dark cycle (Vaulot et

al. 1995, Liu et al. 1997, Shalapyonok et al. 1998). This allows one to determine the

cell division rate by analyzing DNA histograms taken over the light dark cycle. The

ability to distinguish cyanobacteria from other phytoplankton, and to accurately

determine the cell division rate of *Prochlorococcus* allows one to ask subtle

questions about cyanobacteria physiology and ecology.

Low concentrations of essential trace metals can influence the biochemistry of

individual cells and the composition of phytoplankton populations. Trace metals

concentrations in the open ocean can be orders of magnitude lower than in coastal

regions. For instance, total iron concentrations are 300 fold higher close to the coast

than in the open ocean (Martin and Gordon 1988). In response the biochemistry of oceanic phytoplankton has evolved to require lower cellular quotas for Fe, Mn and Zn (Brand et al. 1983). For example, iron limited diatoms can substitute flavodoxin for the Fe containing electron transport protein ferredoxin (La Roche et al. 1996). The equatorial Pacific is a high nutrient, low chlorophyll area where iron concentrations are low. Under these conditions larger eukaryotic cells are rare, even though their cellular chemistry is finely tuned to survive with a low cellular quota for iron. The ecosystem is dominated by small cells (Cavender-Bares et al. 1998, Appendix A), which have greater surface area to volume ratios and are less likely to become diffusion limited (Morel et al. 1991b). Are the small picoplankton, as well as the larger eukaryotic cells, limited by iron in the equatorial Pacific? This question was addressed by determining the cell division rates of *Prochlorococcus* in the equatorial Pacific before and after an *in situ* addition of iron (Chapter 2).

Trace metals can also impact cyanobacteria ecology by being present in toxic, rather than limiting concentrations. Cu is an essential trace element that is present in plastocyanin and cytochrome oxidase and is used as an electron carrier in both photosynthesis and respiration (Baron et al. 1995). However, free cupric ions (free Cu^{2+}) in pM quantities are toxic to cyanobacteria, which are very sensitive to the trace metal environment. In high concentrations copper decreases photosynthesis by inhibiting PSI electron transport and interfering with PSII photochemistry (Baron et

al. 1995). Copper can also disrupt photosynthetic pigment synthesis. For example, the percent of chlorophyll a allomer increases with Cu concentration in *Phaeodactylum tricornutum* (Cid et al. 1995). Changes in membrane permeability leading to a loss of intracellular potassium are associated with copper toxicity in the dinoflagellate *Prorocentrum micans* (Lage et al. 1996). Copper also binds to -SH groups in enzymes thereby disrupting enzyme function (Brown et al. 1994, Stauber and Florence 1987). Further enzyme inactivation and membrane damage can occur when the reduction of Cu(II) by glutathione produces Cu(I), which can react with H_2O_2 to produce hydroxyl radicals (Brown et al. 1994). Finally, high copper concentrations can interfere with the uptake of other essential trace metals (Sunda and Huntsman 1983).

The concentration of Cu and other trace metals in the Sargasso Sea is relatively high. Total dissolved copper in the northern Pacific and the subtropical northeastern Atlantic ranges from 0.5 to 1.5 nM at the surface. The concentration of organic Cu binding ligands (L_1) in both areas exceeds the concentration of dissolved copper, so free Cu^{2+} concentrations at the surface are low, from 0.01 to 0.2 pM at the surface (Buckley and Van Den Berg 1986, and Coale and Bruland 1990). In contrast, in shallow mixed layers in the northern Sargasso Sea the concentration of copper is higher than that of L_1. As a result, free Cu^{2+} concentrations can reach 6 pM, 30 to

600x greater than those in the Pacific or northeastern Atlantic (Moffett 1995 and Moffett et al. 1990).

When the water column in the Sargasso Sea becomes stratified in the summer, there is a rough inverse correlation between *Prochlorococcus* abundance and free Cu^{2+} concentrations such that cell numbers are low in shallow mixed layers (Olson et al. 1990) where free Cu^{2+} is high (6 pM) (Moffett 1995 and Moffett et al. 1990) and higher below the mixed layer where the copper concentration is lower (Olson et al. 1990). The distribution of *Synechococcus* is more uniform with depth (Olson et al. 1990). This pattern of relative cyanobacteria abundance in the Sargasso Sea are consistent with the hypothesis that cupric ion activity (free Cu^{2+}) may play a role in their ecology. In order to test this hypothesis, the response of cyanobacteria to copper was monitored in the laboratory and in the field.

In the laboratory, the copper sensitivity of *Prochlorococcus* isolates representing both major ecotypes (determined by chl B/A ratios (Moore et al. 1998)) and *Synechococcus* clones isolated from the Sargasso Sea was compared. In the field, three different environments were studied: the Northern Sargasso Sea in January and June when the water column was well mixed and seasonally stratified, respectively and in the permanently stratified Southern Sargasso. These environments represent a range of *in situ* free Cu^{2+} concentrations; with low free Cu^{2+} in the well mixed water

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column and below the thermocline in the stratified water columns and high

concentrations within shallow mixed layers (Moffett, 1995). *In situ* cell division

rates of *Prochlorococcus* with depth and the sensitivity of cyanobacteria to copper

during on deck bottle incubations were determined at each station.

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Chapter 2

Iron limits the cell division rate of *Prochlorococcus* in the Eastern Equatorial Pacific

ABSTRACT

Prochlorococcus, a small unicellular cyanobacterium, is an important member of the phytoplankton community in the eastern equatorial Pacific. When these waters are enriched with iron, the chlorophyll per cell and cell size of *Prochlorococcus* increases, implying that they are iron limited. Their population size, however, remains essentially constant. It has been unclear whether this constancy reflects marginal iron-limitation, or stimulated cell division rates which have been matched by equally elevated grazing rates. To examine this, we used a cell cycle analysis approach to measure cell division rates directly, both in and out of the Fe-enriched patch, and in Fe-enriched bottles. The cell division rate increased from 0.6 to 1.1 day^{-1} over six days of exposure to the elevated iron concentrations in the patch. Cells incubated in bottles with additional iron had rates of 1.4 day^{-1}, or two doublings per day. *Prochlorococcus* mortality rates, measured independently, nearly doubled after the addition of iron. This increase in mortality essentially matched the increase in the cell division rate, thus maintaining a relatively constant population size.

INTRODUCTION

In the equatorial Pacific, nitrate and phosphate concentrations are persistently high in the euphotic zone and phytoplankton biomass and productivity are lower than expected based on nutrient availability (Cullen 1991, Frost and Franzen 1992). The phytoplankton community is dominated by small picoplankton (Chavez 1989), which have a large surface area to volume ratio and are less likely to be diffusion limited by either nutrients or trace metals than larger cells (Morel et al. 1991a). Measured cell division rates are often 0.5 day^{-1} or higher, both for the phytoplankton community as a whole (Barber 1991, Chavez et al. 1991, Cullen 1991, Cullen et al. 1992, Landry et al. 1995, Verity et al. 1996, Latasa et al. 1997, Lindley 1998) and for the small cyanobacterium *Prochlorococcus* (DuRand 1995, Landry et al. 1995, Vaulot et al. 1995, Binder et al. 1996, Latasa et al. 1997, Liu et al. 1997, Vaulot 1999). These are relatively high values, suggesting that the dominant components of the community are growing at near maximal growth rates. Estimated ratios of new production to primary production are low (McCarthy et al. 1996) indicating that most of this growth is fueled by regenerated nutrients, which are produced by the intensive grazing that keeps the picoplankton community in check (Wheeler and Kokkinakis 1990, Chavez et al. 1991, Cullen et al. 1992, Frost and Franzen 1992, Price et al. 1994, Landry et al. 1997).

Why is the equatorial Pacific dominated by small cells growing on regenerated nutrients, when the persistently high concentrations of nitrate and phosphate could support new production and the growth of larger phytoplankton? The iron hypothesis explains these observations by maintaining that phytoplankton productivity and biomass are limited by iron (Martin et al. 1991, Coale et al. 1996b). Ambient iron levels are low (Coale et al. 1996b), and the addition of iron to incubation bottles preferentially stimulates the net growth rate, chlorophyll yields, nitrate uptake, and nutrient consumption of larger cells (Chavez et al. 1991, Martin et al. 1991, Price 1991, Price et al. 1994, Takeda and Obata 1995, Fitzwater et al. 1996). The relatively small surface to volume ratio of large cells makes them competitively inferior for NH_4^+ and Fe assimilation because of diffusion limitation (Morel et al. 1991a). Nitrate concentrations are high because of iron limitation, particularly of the large diatoms, and because the intensive grazing of smaller phytoplankton sustains relatively abundant NH_4^+ concentrations which preempt the consumption of NO_3^- (Wheeler and Kokkinakis 1990, Price et al. 1994, McCarthy et al. 1996, Landry et al. 1997, Loukos et al. 1997). Iron enrichment significantly stimulates the assimilation of NO_3^- — but not that of NH_4^+ (Price et al. 1994) — which is consistent with the observation that nitrate reduction requires large amounts of Fe (Raven 1988).

Results from two *in situ* iron fertilization experiments have confirmed the importance of iron limitation in the equatorial Pacific. Addition of approximately 4 nM iron during IronEx I resulted in a three fold increase in chlorophyll *a* and productivity (Martin et al. 1994). The response during IronEx II, in which three pulses of iron were added, was even greater. Total chlorophyll increased at least 12-fold from 0.2 to 2.6 μg liter^{-1} 6 days after the iron addition (Cavender-Bares et al. 1998) and may have increased as much as 27-fold (Coale et al. 1996b). At the same time, the nitrate concentration decreased by approximately 5μM (Coale et al. 1996b).

A major portion of the biological signal in iron enrichment experiments is attributable to large phytoplankton, especially diatoms (Chavez et al. 1991, Price et al. 1994, Takeda and Obata 1995, Fitzwater et al. 1996, Zettler et al. 1996). During IronEx II, chlorophyll *a* in the >10μm fraction increased 60-fold, and the bulk diatom pigment concentration and pennate diatom abundance increased significantly (Coale et al. 1996b, Cavender-Bares et al. 1998). During the first 4 days after iron addition, pennate diatoms had a net growth rate (i.e. measured increases in cell number) of 1.0 day^{-1} (Cavender-Bares et al. 1998). Since the grazing rate of the mesozooplankton did not decrease, the cell division rate of the diatoms must have increased dramatically (Coale et al. 1996b). Finally, cells >5μM accounted for 85 to 98% of the total biomass specific nitrate uptake, which increased 5 to 7 fold as a result of iron fertilization (Coale et al. 1996b).

There are also some indications that the cell division rates of the picoplankton in the equatorial Pacific might be significantly iron limited in spite of their small size and relatively high growth rates. *Synechococcus*, a close relative of *Prochlorococcus*, is known to have a relatively high demand for iron (Brand 1991). Moreover, the quantum yield of the total phytoplankton community, which is dominated by picoplankton, is low in this region because of iron limitation (Greene et al. 1994, Kolber et al. 1994, Lindley et al. 1995, Behrenfeld et al. 1996, Kudela and Chavez 1996), and models predict that an increase in iron supply would cause an increase in productivity (and by inference cell division rate) (Lindley et al. 1995, Chai 1996). In addition, Price et al (1994) concluded that the indigenous (picoplankton dominated) population is iron stressed because the short term iron uptake rates measured in the equatorial Pacific are rapid and comparable to rates seen in iron limited cultures in the laboratory. Finally, dilution series (Landry et al. 1995) carried out during IronEx II showed that the division rate of cells in the $<5\mu M$ fraction doubled in the + Fe patch (Coale et al. 1996b).

In order to assess the degree of iron limitation of the indigenous picoplankton community during IronEx II, we focused our analysis on *Prochlorococcus*. As the smallest and most abundant component of the phytoplankton, it is likely to be the least iron stressed member of the community. We observed that *Prochlorococcus*

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cell volume and chlorophyll *a* per cell increased after the addition of iron during

IronEx II, but cell numbers remained constant over the course of the experiment

(Cavender-Bares et al. 1998). There are two possible explanations for this; either

Prochlorococcus was slightly iron stressed, but already growing close to its maximal

rate so the addition of iron did not have a significant effect on the cell division rate,

or cell division rate did increase significantly in response to the Fe-additions, but the

grazing rate increased commensurately, keeping the population levels constant. The

latter is possible because microflagellate predators of small picoplankton can grow as

fast as, or faster than, their prey (Banse 1982). Without independent measures of cell

division rate and grazing rate, it is impossible to distinguish between the two

possibilities outlined above.

To this end, cell division rates of *Prochlorococcus* were calculated using DNA

histogram analysis before and after the *in situ* addition of iron during the IronEx II

experiment. Cells from the + Fe patch were also incubated on deck in bottles which

contained additional iron. These cell division rates were compared with

Prochlorococcus specific mortality rates measured using the dilution technique (M.

Landry, pers. comm.).

METHODS

Sample collection and preservation. In the 1995 IronEx II experiment, three pulses

of iron were added to the Equatorial Pacific (4°S, 105°W) on days 0, 3, and 7 in

order to increase the iron concentration from 0.05 nM to 1.0 to 2.0 nM (Coale et al.

1996b, Rue and Bruland 1997). The cell cycle analysis we employed in this study

requires high frequency sampling over 24 hours. This was done during a site survey

to characterize the area before iron was added, and on day 6 inside the iron fertilized

patch. For our purposes, the site survey data served as a control for the iron

fertilized patch because it was impossible to do 24 hour sampling programs

simultaneously in and out of the patch. During each diel sampling program, samples

were collected approximately every 30 to 60 minutes from the ship's clean flow

through seawater system (intake at 6m). Given the multi-investigator nature of the

IronEx II study it was not possible to do high frequency depth profile sampling.

In order to obtain more information on *Prochlorococcus* growth and diel patterns

than was possible from *in situ* sampling, samples were also taken from bottle

incubations. These were initiated on the morning of day 5 using 15m water collected

from the patch with a trace metal clean Go-Flo bottle (Fitwater 1982). Two bottles

were placed in an on deck incubator which simulated the *in situ* light and

temperature (bottles received approximately 40% of the irradiation at 0.5m and were

cooled with surface seawater) and sampled every hour for 48 hours. In spite of trace metal clean precautions in setting up this experiment, the measured iron concentration in the bottles was 1.23 nM, at least 3.6x the concentration inside the patch (M. Gordon, personnel communication). The results from this analysis, hereafter referred to as the "+Fe bottles", were used to determine the growth rate of *Prochlorococcus* at iron concentrations higher than those in the patch, rather than as a proxy for additional *in situ* data as was originally intended. All samples were preserved in a final concentration of 0.1% glutaraldehyde for 10 minutes at room temperature in the dark and were then stored in liquid nitrogen (Vaulot et al. 1989).

DNA staining with Sybr Green 1. To eliminate interference from RNA, thawed samples were incubated with RNase A (Sigma-Aldrich R-4875) for 30 minutes at 37°C at a final concentration of 1 µg/ml. Potassium citrate (final concentration 30 µM) and a $1:10^{-4}$ final dilution of the DNA and RNA specific stain Sybr Green I (Molecular Probes) were then added, and the samples were maintained at room temperature in the dark for 15 minutes before analyzing by flow cytometry. All working stocks of Sybr Green I were diluted in DMSO (Marie et al. 1997).

The amount of DNA per cell, as well as *Prochlorococcus* number and forward angle light scatter (FALS, which is a measure of cell size), were quantified using a modified EPICS 753 flow cytometer as described in Binder et al (1996) except the

instrument was used in single beam mode and the optical filters were arranged to detect green fluorescence from Sybr Green I, which is excited at 488 nm. After passing through 630 and 560 nm short-pass dichroic filters, green signals passed though a 515 nm long-pass filter. Samples were delivered at a constant rate using a syringe pump (Harvard Apparatus, Model 22). The lowest coefficients of variation (CV) in terms of fluorescence were achieved by using low sample flow rates (5 μl min^{-1}) and sample lines pre-equilibrated with the stain for at least one hour.

Calculating the cell division rate:

Cell cycle analysis method. The cell cycle of *Prochlorococcus* is similar to the eukaryotic cell cycle in that it has a discrete DNA synthesis phase, even when the cells are growing faster than one division per day (Shalapyonok et al. 1998). As a result the eukaryotic terms G_1, S, and $G_2 + M$ are often applied to the *Prochlorococcus* cell cycle for convenience. Moreover, cell division in *Prochlorococcus* is tightly synchronized to the light dark cycle (Vaulot et al. 1995, Liu et al. 1997, Shalapyonok et al. 1998), which allows one to estimate the duration of the S and G_2+M phases of the cell cycle and apply the DNA histogram method of estimating cell division rates (McDuff and Chisholm 1982, Carpenter and Chang 1988, Vaulot et al. 1995, Liu et al. 1997). In order to determine the duration of S and G_2+M, DNA fluorescence histograms were modeled and divided into G_1, S, and G_2+M using ModFit software (Verity Software House, Inc.). Data from at least

4,000 cells were collected per sample in order to ensure accurate modeling of the

DNA histograms. The average CV of the G_1 peak was 6.8%, with a standard

deviation of 2.2%.

Following the method of Liu et al. (1997), the cell division rate (μ day^{-1}) was

obtained from the DNA histograms using

$$\mu = \frac{1}{t_{(S+G_2)}} \int_{0 \text{ hrs}}^{24 \text{hrs}} \ln[1 + f(S+G_2)_t] \qquad (1)$$

Where $f(S+G_2)_t$ is the fraction of cells in the S and G_2+M phases of the cell cycle at

time t, and $t_{(S+G_2)}$ is the time (in hours) required for cells to pass through S and G_2 +

M. The latter can be estimated as twice the distance between the peak of cells in the

S phase and the peak of cells in the G_2+M phase (Carpenter and Chang 1988). Using

this approach the $t_{(S+G_2)}$ values for the site survey, inside the patch, and the +Fe

bottles were 4.3, 2.3, and 3.0 hours (for both bottles), respectively (Table 1). $t_{(S+G_2)}$

can also estimated as the time between the beginning of S phase and the first increase

of cell number or cells in G_1 (Shalapyonok et al. 1998); the results using this method

agree well with those given above (Table 1). All of these values are significantly

shorter than the 6 hours used by Vaulot et al. (1995) and Liu et al. (1997) for

Prochlorococcus in the equatorial Pacific and the subtropical north Pacific,

respectively. We suspect that this is partially a result of the higher frequency

sampling program used in our study, which allows greater resolution of cell cycle

phase durations (McDuff and Chisholm 1982, Carpenter and Chang 1988). Cell

cycle duration times shorter than 6 hours have also been found for *Prochlorococcus*

in the Arabian Sea (2 to 3 hours, (Shalapyonok et al. 1998)).

Cell number method. The cell division rate can also be calculated if the net specific

growth rate (k, day^{-1}) and mortality rate (m, day^{-1}) are known. The net growth rate

was calculated as

$$k = \frac{\ln N_1 - \ln N_0}{t} \qquad (2)$$

where N_0 and N_1 are the numbers of cells at time 0 and one day later, respectively

and t is one day. *Prochlorococcus* mortality rates, m, were calculated from the slope

of the decrease in abundance over the time interval when the cells were not dividing

(Vaulot et al. 1995, Liu 1998). This method assumes that mortality remains constant

throughout the day, and in some cases this is clearly not true. For instance, the

decline in *Prochlorococcus* numbers during the site survey was faster from 24:00 to

6:00 than from 6:00 to 15:30 (Fig 1). Despite such deviations from linearity,

mortality rates calculated in this manner can be helpful in bounding the problem,

thus we include them here. Once values of m and k are at hand, the cell division rate

can then be determined as

$$\mu = k + m \qquad (3)$$

Glossary of important terms

Parameter	Units	Definition
μ	day^{-1}	Cell division rate: the growth rate in the absence of cell losses
k	day^{-1}	Net growth rate: cell division rate minus cell mortality rate
m	day^{-1}	Mortality rate: cell losses
$f(S+G_2)_t$	unitless	Fraction of cells in the S and G_2+M phases of the cell cycle at time t
$t_{(S+G_2)}$	days	Duration of the S and G_2+M phases

RESULTS AND DISCUSSION

Cell cycle progression patterns

The progression of *Prochlorococcus* through the cell cycle and the degree of synchronization during the site survey (the "no iron added" control) were similar to previous data from the equatorial Pacific (Vaulot et al. 1995, Liu et al. 1997). Cell division was tightly synchronized to the light dark cycle (Fig 1A, B, and C), with cells remaining in G_1 during most of the day, indicating that there was no cell division during this period (Fig 1C). As a result of grazing and other losses, cell numbers declined during the day (Fig 1A). At the same time the relative mean FALS per cell increased as the cells grew in size during G_1 and S reaching a maximum at 19:00 (Fig 1B). DNA synthesis started at approximately 16:40 and by 19:00 75% of the cells were in the S phase and a few had progressed to G_2+M (Table 1). The peak of cells in G_2+M and the first increase in cell numbers occurred two hours later, followed by a decrease in FALS per cell as the cells divided. (Fig 1B and C).

A qualitative comparison of *Prochlorococcus* cell cycle patterns in the site survey, + Fe patch, and + Fe bottles revealed significant differences that are consistent with the hypothesis that iron addition stimulated cell division rates. In all populations, we

Fig 1) Diel patterns seen in *Prochlorococcus* collected from 6m during the site survey. The solid bar represents the dark period, vertical dotted lines point out times of interest. A) Cell concentration. The dashed line represents the regression used to calculate the mortality rate (see text). B) Mean forward angle light scatter (FALS) normalized to standard beads. C) The fraction of total *Prochlorococcus* in each cell cycle stage; DNA is synthesized during the S phase, G_1 is the gap before DNA synthesis takes place and G_2+M includes the gap after the S phase as well as mitosis. D) Selected DNA histograms from the analysis, note the persistence of a substantial G_1 peak throughout the day.
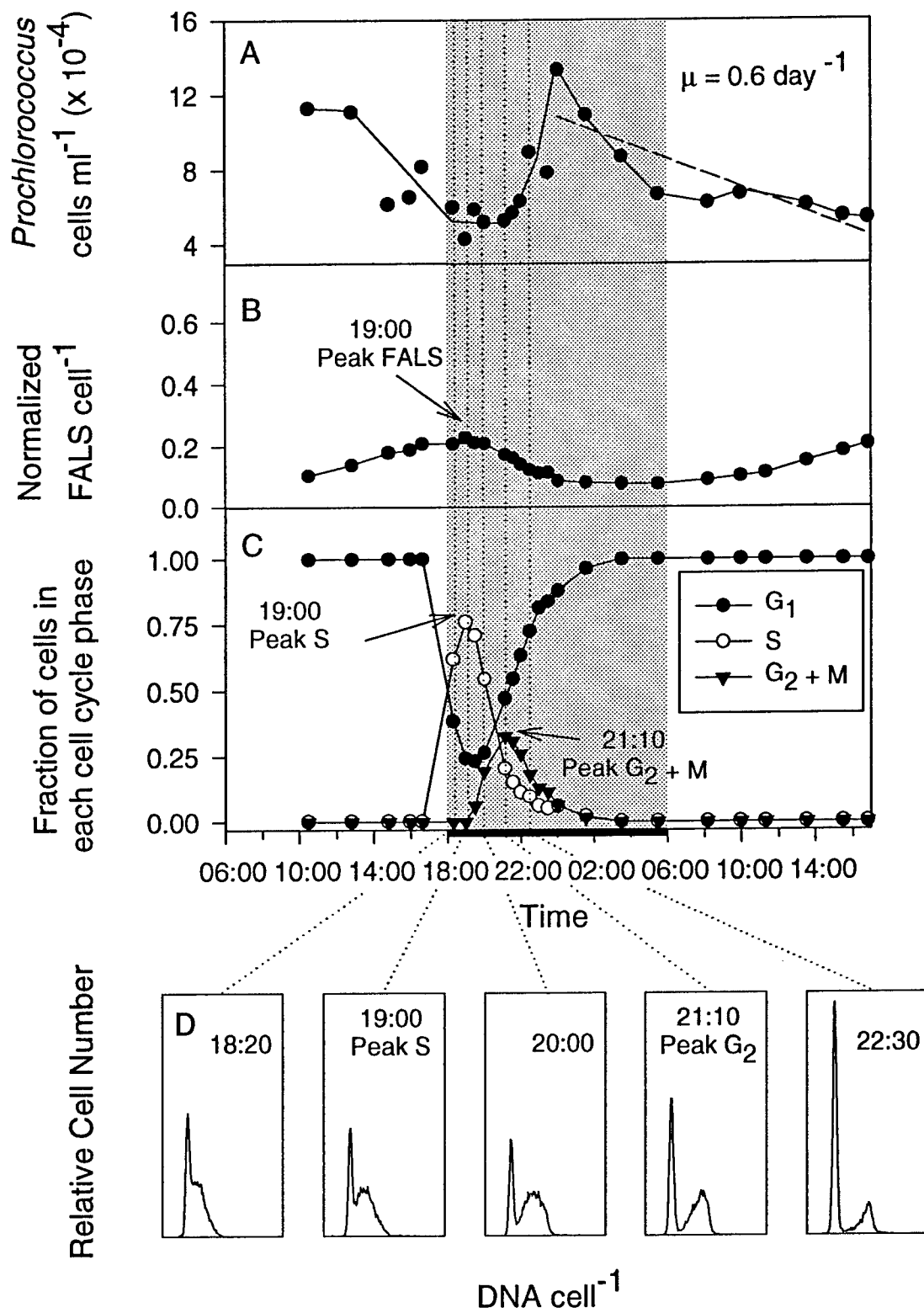
Table 1. The timing of cell cycle progression and values of critical parameters used in cell division rate calculations for *Prochlorococcus* populations in and out of the patch, and in Fe-enriched bottles.

Cell Cycle Parameters	Site Survey	Inside Patch	+Fe Bottles
S phase initiation	16:40	16:00	16:30
S phase peak	19:00	17:50	17:30
G_2+M phase initiation	19:00	17:30	17:30
G_2+M phase peak	21:10	19:00	19:00
First increase of cells in G_1	20:00	18:10	18:30
First increase in cell number	21:10	18:10	ND
FALS max/min ratio	3.0	3.6	4.6
$t_{(S+G_2)}$, hours	4.3	2.3	3.0

saw the classic diel patterns reflecting synchronized cell division in *Prochlorococcus* populations (Vaulot et al. 1995, Binder et al. 1996, Vaulot 1999). These are characterized by cell abundance decreases (due to grazing in the absence of cell division) and FALS per cell increases (reflecting increases in cell volume and biomass) during the day, with a reversal of these trends at night when cell division takes place (Fig 1A,B, 2A,B, 3A,B). We found, however, that the maximum FALS

Fig 2) Diel patterns seen in *Prochlorococcus* collected from 6m inside the iron fertilized patch. Samples from 16:30 to 21:00 were run in duplicate, error bars represent one standard deviation and are occasionally smaller than the symbols. The solid bar represents the dark period, vertical dotted lines point out times of interest. Panels as in Fig 1. Note the lack of a substantial G_1 peak at 17:30 and 18:10 and the large changes that occurred between 18:10 and 18:30.
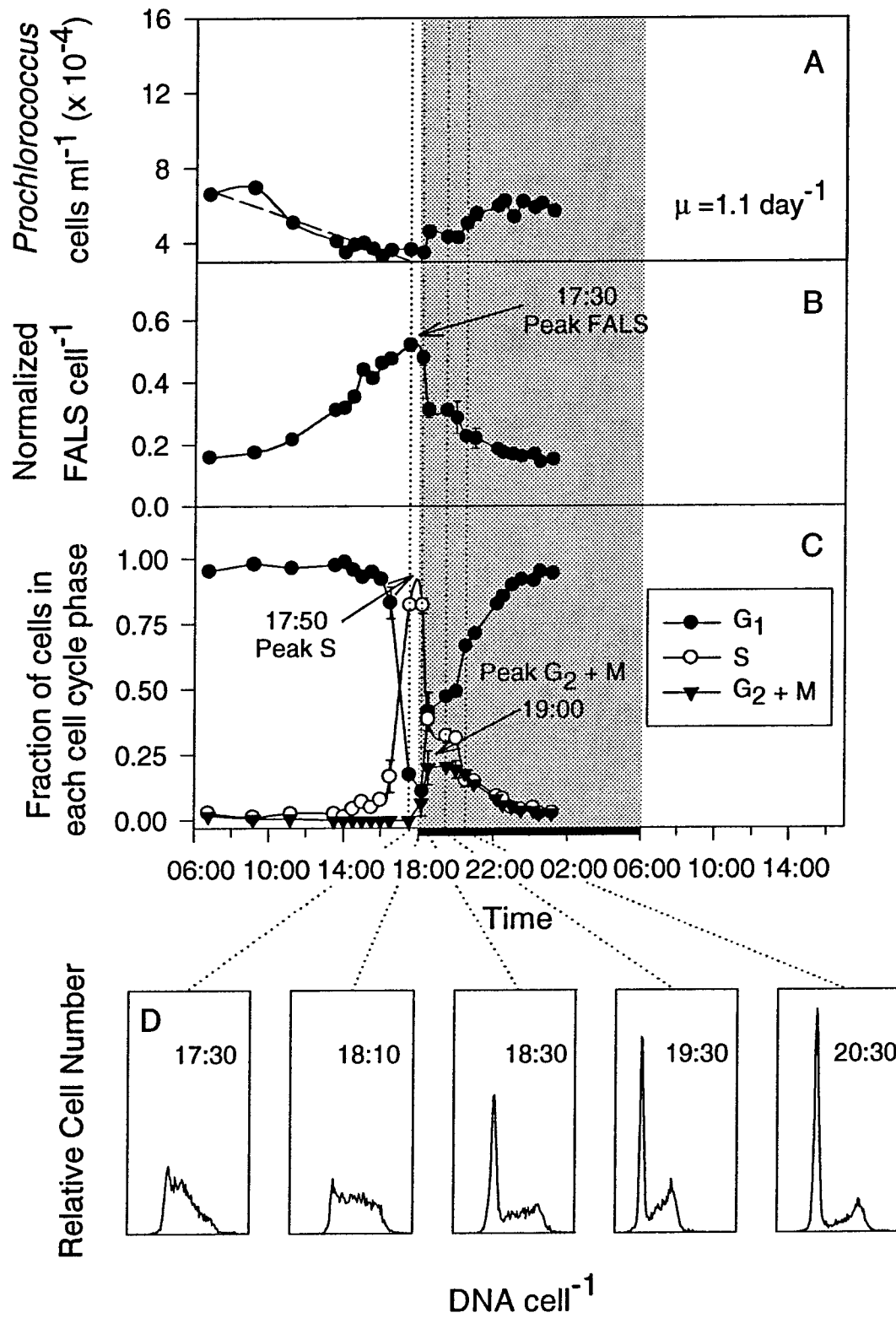
Fig 3) Diel patterns seen in *Prochlorococcus* in the +Fe bottles. The concentration

of iron in these bottles was 1.23 nM, approximately 3.6 times the concentration

found inside the patch. The solid bar represents the dark period, vertical dotted lines

point out times of interest. Panels as in Fig 1. Note the lack of a substantial G_1 peak

at 17:30 and the second peak of *Prochlorococcus* in both S and G_2+M.
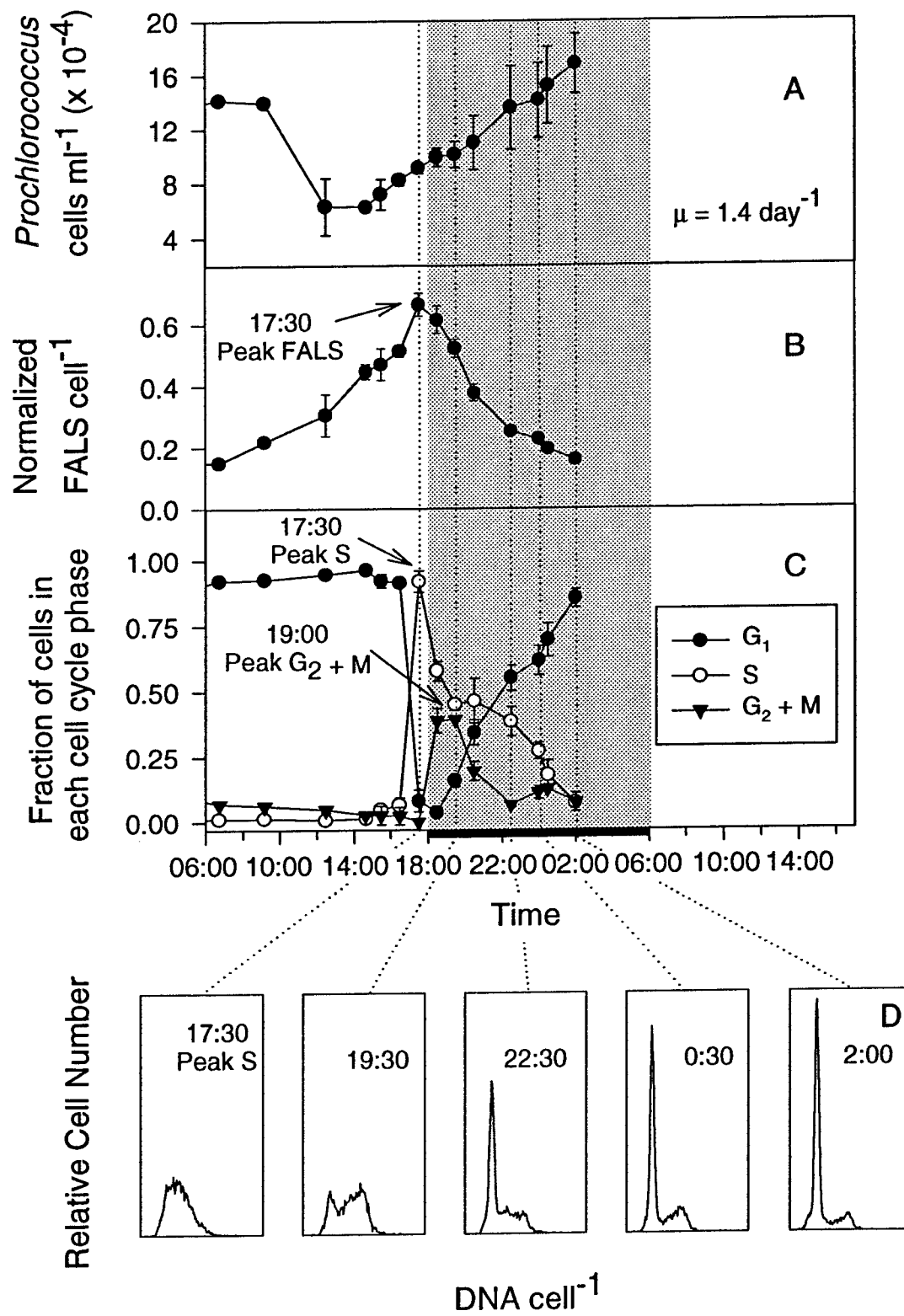
per cell ($FALS_{max}$) and the amplitude of the FALS change over the diel cycle ($FALS_{max}/FALS_{min}$) were significantly higher in populations exposed to elevated iron levels. The $FALS_{max}$ for the site survey population was 0.23, whereas it was 0.52 for the population inside the patch, and 0.66 for the cells in the +Fe bottles (Figs 1B, 2B and 3 B). The $FALS_{max}/FALS_{min}$ ratio also increased with increasing iron concentration, from 3.0 to 3.6 to 4.6 for the site survey, +Fe patch, and +Fe bottles, respectively (Table 1). If cell division was synchronized to the same extent in all of the groups, the cells with the greatest change in FALS ($FALS_{max}/FALS_{min}$) would have the highest cell division rate (DuRand 1995, Binder et al. 1996). The ratio of 3.6 inside the patch is comparable to that observed by Binder (1996) for equatorial Pacific waters, whereas the value of 4.6 observed in the bottles - reflecting an 80% change in mean FALS per cell over the diel cycle - is significantly higher than any previous data from the equatorial Pacific (DuRand 1995, Binder et al. 1996).

If we now look at the actual progression through the cell cycle stages in the three populations, the differences are even more apparent (Fig 1C, 2C, and 3C; Table 1). Progression through the S and G_2+M phases was much faster in the populations that had added Fe ($t_{(S+G_2)}$ = 2.3, and 3.0 h for the +Fe patch and the +Fe bottles, respectively) compared to that of the site survey population ($t_{(S+G_2)}$ = 4.3h) (Table 1). The timing of cell division was also shifted one to two hours earlier in the day in the +Fe populations (Fig 1C, 2C, and 3C; Table 1).

Finally, it can be seen clearly from the cell cycle progression data that the cells in the patch and in the +Fe bottles underwent a second round of division over a 24 hour period, whereas those during the site survey did not (Figs 1C,D, 2C,D, and 3C,D). For a population to have a division rate greater than 0.69 day^{-1} (= 1 doubling day^{-1}) some cells must divide more than once per day. Because *Prochlorococcus* is tightly phased to the light dark cycle, the second division occurs after the first division round is complete, and manifests itself in the DNA histograms as a second peak in the S and G$_2$+M phases (Shalapyonok et al. 1998). This second round of division was observed only when Fe was added (Compare Figs 1C,D; 2C,D). The first division burst inside the patch, which can be seen clearly in all of the cell cycle parameters (Fig. 2 A,B,C,D), occurred between 18:10 and 18:30. The second division event, which was smaller, occurred after 20:00, when there was a second drop in the proportion of cells in S phase accompanied by an increase in the number of cells in G$_1$ and a continuing decrease in FALS (Fig 2A, B, and C). One division between 18:10 and 18:30 followed by a second division after 20:00 would require a progression through the first cell cycle of approximately two hours, which is close to the estimate of 2.3 hours for t$_{(S+G_2)}$ (Table 1). DNA histograms of *Prochlorococcus* from the +Fe bottles also indicate that these cells were growing much faster than one doubling per day, and had a second round of division (Fig. 3). In these populations, the first peak in S phase occurred at 17:30 and the second could be seen clearly at 20:30 (Fig 3 C). Two peaks in G$_2$+M were also evident (Fig 3C).

Cell Division Rates: Cell cycle analysis

Using equation (1), the $t_{(S+G_2)}$ values in Table 1 and the cell cycle data in Figs 1C,

2C, and 3C, we were able to calculate cell division rates for the three

Prochlorococcus populations growing at different levels of iron. In the + Fe

populations it was assumed that the $t_{(S+G_2)}$ values for the first and second rounds of

cell division were identical. The cell division rate determined for the site survey

using cell cycle analysis was 0.6 day^{-1} (Table 2), in agreement with previous

estimates (0.5 to 0.7 day^{-1}) from similar depths for this region (DuRand 1995,

Vaulot et al. 1995, Binder et al. 1996, Latasa et al. 1997, Liu et al. 1997). The

division rate in the Fe enriched patch was 1.1 day^{-1}, almost double that calculated

for the site survey cells (Table 2). Populations in the +Fe bottles had cell division

rates of 1.4 day^{-1}, which is equal to two doublings per day and significantly higher

than any growth rate previously recorded for *Prochlorococcus* in the equatorial

Pacific. Division rates greater than one division per day that are either supported by

a second round of division seen in the DNA histograms, or calculated by the dilution

technique (Landry et al. 1995) have only been reported for the highly productive

Arabian Sea (Reckermann 1997, Liu 1998, Shalapyonok et al. 1998).

One could argue that the second division burst observed in the Fe-enriched

populations was due to a second *Prochlorococcus* population which was

synchronized differently to the light/dark cycle. Although not seen in our samples from the equatorial Pacific, dual populations have been documented (Campbell and Vaulot 1993) and if this were the case here, our growth rates could be overestimated. We think this is unlikely, however, for the following reasons: First, similar behavior – ie. a second round of division – has also been observed in *Prochlorococcus* cultures isolated from the Sargasso and Arabian Seas (Shalapyonok et al. 1998). Second, cell division rates calculated from cell number data (see below) — which would not be affected by the presence of a second *Prochlorococcus* ecotype — also indicate that two rounds of cell division took place. Finally, we note that the fraction of cells in G_1 goes essentially to zero as the population enters the first round of DNA replication. If there were a second population present waiting to replicate its DNA later, they would still be present in G_1 at this point and the fraction of *Prochlorococcus* in this cell cycle stage would be a significant fraction of the cells in the second S phase peak, which is close to 40%.

Cell Division Rates: Cell Number, Net Growth Rate and Mortality

Cell division rates calculated from changes in population numbers (equations 2 and 3) are in good agreement with results based on cell cycle analysis (Table 2). Using the net change in population numbers to calculate the net growth rate, k (equation 2), we found the values were –0.4 ± 0.2 and –0.2 during the site survey, and in the

patch, respectively. Net growth rates were determined using the difference in cell numbers between days 6 and 7. In this time interval there were three pairs of time points approximately 24 hours apart in the site survey (15:30 to 16:00, 16:50 to 16:40, and 10:00 to 10:30), this data was averaged to calculate the net growth rate. Data for inside the patch was less extensive, the net growth rate was estimated using the difference in cell numbers from 6:45 on day 6 to 1:10 on day 7, which should be long enough to eliminate diel effects (see Fig. 1A). Because this does not represent a full 24 hour period, however, the net growth rate could be overestimated (which will also lead to an overestimate of the cell division rate).

Mortality increased in parallel with the cell division rate, the ability of grazers to balance picoplankton cell division rates and prevent these small cells from increasing in abundance has been noted by several researchers (Chavez et al. 1991, Cullen 1991, Frost and Franzen 1992, Cullen 1995, Verity et al. 1996, Loukos et al. 1997). In particular, *Prochlorococcus* cell division rate and mortality are tightly coupled in the equatorial Pacific (DuRand 1995, Vaulot et al. 1995, Binder et al. 1996, Liu et al. 1997). This tight coupling between cell division rate and mortality for the picoplankton is one of the basic tenants of the ecumenical hypothesis (Frost and Franzen 1992, Price et al. 1994) and the equatorial Pacific synthesis by Landry et al (1997). Mortality rates estimated from the *Prochlorococcus* population numbers

Table 2. Summary of *Prochlorococcus* growth and mortality rates (day^{-1}) determined by a variety of methods. Standard deviations around the mean are included in those cases where replicates were present.

	Site Survey	Inside Patch	+ Fe Bottles
Iron Concentration (nM Fe)	0.05	0.05 to 0.34	1.23
Cell Division Rate, μ			
Cell cycle analysis	0.6	1.1	1.4 ± 0.1
Cell number data[d]	0.7	1.3	ND
Mortality Rate, m			
Cell number data	1.0 [a]	1.7 [a]	1.2 ± 0.1 [b]
Dilution method[c]:	<u>0.5</u>	<u>1.2</u>	ND
Mean	0.75	1.5	
Net Growth Rate, k			
Cell number data	-0.4 ± 0.2	-0.2	0.2 ± 0.1

[a] **Mortality rate calculated from the decline in cell numbers when no cell division was taking place.**

[b] Mortality rate calculated from the net growth rate and the cell division rate based on cell cycle analysis.

[c] M. Landry, pers. comm.

[d] Calculated using the mean mortality rate.

were 1.0 and 1.7 day^{-1}, in the site survey and in the iron patch, respectively. Cell number data from midnight to 16:50 in the site survey and 6:45 to 17:30 in the iron patch were used to calculate these mortality rates. Mortality rates estimated from the dilution technique (Landry pers. com.) were similar to the cell number results at 0.5 and 1.2 day^{-1}. Averaging the two estimates of mortality yields values of m = 0.75 and 1.5 day^{-1} for the site survey and patch. It is not surprising that mortality rates increased in the +Fe patch since the growth and grazing rate of heterotrophic protists that feed on picoplankton can be iron limited (Chase and Price 1997). Finally, applying equation 2 yields cell division rates of μ = 0.7 and 1.3 day^{-1} for these populations, which is in agreement with the values calculated from cell cycle analyses (Table 2).

The cell number data from the bottle incubation experiments was not adequate to calculate m from the decrease in cells during the day. Thus for these experiments we had to rely on our measurements of μ from the cell cycle analysis, and values of k from the cell number data to calculate m, which was 1.2 day^{-1}. This is similar to the mortality rates seen *in situ,* an indication that confinement in bottles did not significantly effect *Prochlorococcus* grazers.

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Prochlorococcus productivity

We can estimate the contribution of *Prochlorococcus* to primary productivity in and

out of the patch using the following equation:

$$P = C_{cell}N[e^{\mu(t)-1}] \qquad (4)$$

where P is the amount of carbon assimilated by *Prochlorococcus* in mgC m^{-3} day^{-1},

C_{cell} is the carbon per cell, N the number of *Prochlorococcus* cells per m^3 and μ the

cell division rate, day^{-1} (Eppley 1968). We used a C_{cell} value of 53 fg cell^{-1} for cells

during the site survey (Campbell and Nolla 1994, Vaulot et al. 1995), and 85 fg cell^{-1}

for the cells inside the patch. This increase was based on our observed 1.6 fold

increase in *Prochlorococcus* cell volume inside the patch relative to the site survey

(Cavender-Bares et al (1998), assuming carbon increased proportionally with

volume. *Prochlorococcus* population number data (N) were taken from Cavender-

Bares et al (1998), as were values of chlorophyll per cell. Values for the former

were 1.7 x 10^5 (the average of cell numbers during days 4 and 8) and 8.7 x 10^4 (from

day 6) during the site survey and in the patch, respectively.

Using these values and the cell division rates measured using cell cycle analysis, the

amount of carbon fixed by *Prochlorococcus* during the site survey was calculated to

be 5.8 mgC m^{-3} day $^{-1}$ whereas after 6 days in the iron enriched patch it had increased to 9.0 mgC m^{-3} day $^{-1}$. The comparable increase in primary productivity for the entire phytoplankton community in the patch was from 17.8 to 162 mgC m^{-3} day $^{-1}$ over this period (D. Barber pers comm). Thus the relative contribution of *Prochlorococcus* to total primary production decreased from 33% to 6% as the phytoplankton community structure shifted to one dominated by larger cells.

The increase in *Prochlorococcus* productivity resulted from changes in cellular performance, not changes in population size (which actually decreased inside the iron fertilized patch). Carbon fixation per cell and per unit chlorophyll increased by 3.0 and 1.2 fold, respectively. Chlorophyll specific productivity did not increase significantly because the carbon to chlorophyll ratio decreased as the cells synthesized more chlorophyll in response to the addition of iron (Cavender-Bares et al. 1998). While these estimates of *Prochlorococcus* productivity are rough, they parallel changes in productivity of the phytoplankton community as a whole during IronEx I where the productivity per unit chlorophyll did not increase upon the addition of iron because the carbon to chlorophyll ratio of the community decreased (Martin et al. 1994, Lindley 1998).

Photoinhibition, nitrogen limitation and iron.

It is clear that the cell division rate of *Prochlorococcus* increased in the iron-fertilized patch, but it is possible that this was not a direct result of increased iron availability, but rather an indirect response to changes in the ecosystem brought about by the Fe-induced bloom of the larger cells. One could argue, for example, that increased grazing rates in the patch caused elevated NH_4^+ levels, thus stimulating *Prochlorococcus* growth rates. However, NH_4^+ concentrations are high in the equatorial Pacific (0.1 to 0.5 μM). At these concentrations small cyanobacteria, even if they are growing at high cell division rates, can satisfy their nitrogen quota using NH_4^+ without becoming diffusion limited (Morel et al. 1991a, Price 1991, Price et al. 1994). The estimated half-saturation constant for NH_4^+ is twice to ten times lower than the concentrations present (Chai 1996), and in some cases NH_4^+ uptake by the phytoplankton community is saturated (Price 1991, McCarthy et al. 1996). In contrast, half-saturation constants for iron have been estimated as 34 to 120 pM, which is close to or four times higher than the ambient total iron concentration (Price et al. 1994, Coale et al. 1996a). Finally, during both the IronEx I and II experiments NH_4^+ concentrations were consistently lower inside the + Fe iron patch than outside while chlorophyll and productivity increased (Martin et al. 1994 and W. Cochlan pers. comm.). If the phytoplankton were limited by

NH_4^+ before iron fertilization, such a drawdown of NH_4^+ and increase in biomass and productivity would not have been possible.

A second caveat in interpreting our results involves the role of light in regulating cell cycle progression in *Prochlorococcus*. The increase in biomass inside the iron fertilized patch significantly reduced the light intensity available to the cells at 6m as the experiment progressed. The vertical attenuation of light (K_{PAR}) measured by Kludea and Chavez (1996) doubled abruptly between days 5 and 6, which reduced the irradiance at 6m from approximately 70 to 45% of the incident irradiation (Kudela and Chavez 1996). Since the indigenous phytoplankton community at the surface of the equatorial Pacific is photoinhibited (Cullen et al. 1992, Lindley et al. 1995, Vaulot et al. 1995, Liu et al. 1997), release from photoinhibition could have contributed to the increased cell division rates that we observed in the patch.

We are convinced however, that although this could be a contributing factor, it is not the dominant explanation for the increase in cell division rates in the iron-enriched populations. First, independent measures of physiological stimulation in the patch reveal that the initial physiological response – i.e. increased *Prochlorococcus* chlorophyll and FALS per cell, as well as increased photochemical efficiency of the indigenous phytoplankton community was detectable within one or two days of the iron addition – well before light intensity was significantly reduced on day 6 (Kolber

et al. 1994, Behrenfeld et al. 1996, Kudela and Chavez 1996, Cavender-Bares et al. 1998). Second, depth profiles of *Prochlorococcus* cell division rates in the equatorial Pacific revealed that the maximum cell division rate at 30-45m is 30 to 40% more than the cell division rate at the surface, where cells are photoinhibited (Vaulot et al. 1995, Binder et al. 1996, Liu et al. 1997). This difference is not enough to account for the 80% and 130% stimulation in cell division rate we saw in the patch and +Fe bottles, respectively.

CONCLUSION

Despite their small size, which minimizes diffusion limitation (Morel et al. 1991a), *Prochlorococcus* is significantly limited by iron in the equatorial Pacific. When the iron-enriched patch was created in these waters during IronExII, cell size and pigment content per cell increased significantly (Cavender-Bares et al. 1998) reflecting a physiological response to the added iron. We have shown here that the mean cell division rate of *Prochlorococcus* was also significantly stimulated, increasing from one to two doublings per day. This increased growth rate was matched by increased grazing rates, resulting in fairly constant population numbers in the patch. Thus, the low iron concentrations in the equatorial Pacific select for small picoplankton that are efficiently grazed by microheterotrophs and can grow *relatively* quickly (about one division per day) in this high nutrient low chlorophyll region (Barber 1991, Loukos et al. 1997), but which are still significantly iron limited.

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Chapter 3

Copper toxicity and cyanobacteria ecology in the Sargasso Sea.

ABSTRACT

Patterns of relative abundance of *Prochlorococcus* and *Synechococcus* in the Sargasso Sea are consistent with the hypothesis that free cupric ion concentrations (free Cu^{2+}) may play a role in their ecology. For instance, there is a rough inverse correlation between population densities of *Prochlorococcus* and free Cu^{2+} such that cell numbers are low in shallow mixed layers where free Cu^{2+} is high (6 pM) and high below the mixed layer where the copper concentration is low. The distribution of *Synechococcus* is more uniform with depth. In order to test this hypothesis, the response of cyanobacteria to copper was monitored in the laboratory and in the field. The copper sensitivity of *Prochlorococcus* isolates representing both major ecotypes (determined by chl B/A ratios) and *Synechococcus* clones isolated from the Sargasso Sea was compared. *Prochlorococcus* was inhibited at free Cu^{2+} concentrations found in the Sargasso Sea that had no effect on *Synechococcus*. However, high light adapted (low chl B/A) ecotypes were less copper sensitive than low light adapted clones (high chl B/A). In the field, the sensitivity of cyanobacteria was compared during on-deck incubations using water from three different environments: the Northern Sargasso Sea in January and June when the water column was well mixed and seasonally stratified, respectively and in the permanently stratified Southern Sargasso. The effect of copper additions on *Prochlorococcus* from environments where the *in situ* free Cu^{2+} was low (in the well mixed water and below the mixed layer) was significant; the net growth rates were substantially reduced and there was

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a rapid loss in cell numbers. *Prochlorococcus* in shallow mixed layers were less sensitive to copper and may have been members of the copper resistant low chl B/A ecotype. *Synechococcus* were relatively copper resistant across a range of environments in the Sargasso Sea.

INTRODUCTION

Cu is an essential trace element that is present in plastocyanin and cytochrome

oxidase and is used as an electron carrier in both photosynthesis and respiration

(Baron et al. 1995). However, copper can be toxic to phytoplankton in relatively low

concentrations (Brand et al. 1986). Toxic levels of copper can decrease

photosynthesis by inhibiting PSI electron transport and interfering with PSII

photochemistry (Baron et al., 1995). Copper also binds to -SH groups in enzymes

thereby disrupting enzyme function (Brown et al. 1994, Stauber and Florence, 1987).

Further enzyme inactivation and membrane damage can occur when the reduction of

Cu(II) by glutathione produces Cu(I), which can react with H_2O_2 to produce

hydroxyl radicals (Brown et al. 1994). Finally, high copper concentrations can

interfere with the uptake of other essential trace metals (Sunda and Huntsman 1983).

Excess copper is know to be toxic to phytoplankton, but are the ambient levels of

copper in unpolluted environments high enough to actually influence phytoplankton

ecology in the field? Phytoplankton carbon fixation can be reduced by the addition

of copper to natural assemblages in bottle incubations (Fitzwater 1982, Ortner et al.

1984, Sunda 1986, Springer-Young et al. 1992), although this does not occur in

every case (Coale 1991, Cullen et al. 1992). It has also been hypothesized that

phytoplankton growth is inhibited by freshly upwelled water which has a high

copper to manganese ratio (Barber and Ryther 1969, DiTullio and Laws 1991, Sunda and Huntsman 1998b). In addition, environmentally relevant free Cu^{2+} can reduce the cell division rates of phytoplankton isolates in culture. However, no consistent differences in copper sensitivity between phytoplankton clones isolated from oceanic and neritic waters have been found (Brand et al. 1986), even though total copper concentrations can be over an order of magnitude higher close to the coast (Moffett 1995, Kozelka and Bruland 1998). One reason for this is that copper is highly complexed by organic chelators in seawater (Coale and Bruland 1988, Coale and Bruland 1990, Moffett et al. 1990, Moffett 1995, Kozelka and Bruland 1998) and free Cu^{2+} is generally below the concentration that can reduce cell division rates in culture (≤ 1 pM) in unpolluted coastal waters (Moffett et al. 1997, Kozelka and Bruland 1998).

The Sargasso Sea is an ideal environment to extend these studies and determine if copper could influence phytoplankton ecology. For one, in contrast to the comparison between oceanic and coastal waters, there are predictable gradients in free Cu^{2+} with season and with depth in the Sargasso Sea (Moffett et al. 1990). These gradients in free Cu^{2+} are a useful tool because one way to determine if copper toxicity is important is to examine how the phytoplankton community responds to increasing concentrations of copper *in situ*. In the northern Sargasso Sea during the winter the water column is well mixed and the concentration of free Cu^{2+} is low.

Atmospheric deposition of copper decreases during the winter (Moulin et al. 1997, Jickells 1999) and the concentration of the copper binding ligand L_1 is always in excess. As a result, free Cu^{2+} is less than 0.5 pM throughout the water column (Moffett 1995). In the northern Sargasso during the summer, and throughout the year in the southern Sargasso, the water column is well stratified. Under these conditions, free Cu^{2+} changes markedly with depth. Atmospheric deposition of trace metals is higher in the summer and copper accumulates in the shallow mixed layer (Moulin et al. 1997, Jickells 1999). The concentration of L_1 is also lower within the mixed layer than below it, probably because of photodegradation at the surface. As a result, free Cu^{2+} can reach up to 6 pM in the mixed layer but is extremely low in deeper water (Moffett et al. 1990, Moffett 1995).

In addition, a large fraction of the phytoplankton biomass and productivity in the Sargasso Sea is accounted for by the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. *Prochlorococcus* alone can account for 30% of the total chlorophyll, 25% of the yearly primary productivity (Goericke 1993) and 10 to 30% of the total living biomass (Buck K. R. 1996). As a group cyanobacteria are more copper sensitive than eukaryotic cells, possibly because they evolved in an anoxic environment where the concentration of bioavailable copper was low (Brand et al. 1986). The small size and high surface to volume ratio of cyanobacteria, which is an

advantage in acquiring a limiting trace element, may also be a disadvantage if metals are present in potentially toxic concentrations.

There are several indications that copper is likely to play a role in cyanobacteria ecology. For one, *Synechococcus* produces a strong copper binding ligand in concentrations that are regulated by the amount of copper present (Moffett and Brand 1996). This ligand has the same binding constant as the copper binding L_1 found *in situ*, indicating that cyanobacteria may influence trace metal speciation in the oceans (Moffett and Brand 1996). *Synechococcus* populations also decline as free Cu^{2+} increases above 10 pM in polluted harbors (Moffett et al. 1997).

Prochlorococcus abundance also varies with free Cu^{2+}, but at concentrations that indicate that it may be more sensitive to copper toxicity than *Synechococcus*. In stratified water columns in the northern Sargasso Sea there is a rough correlation between high free Cu^{2+} and low *Prochlorococcus* numbers (Moffett 1995). In shallow mixed layers where free Cu^{2+} is expected to be high, *Prochlorococcus* abundance is very low at the surface (Olson et al. 1990). Under these conditions the bulk of the population is present below the mixed layer where the concentration of copper is lower (Moffett 1995). The vertical distribution of *Synechococcus* is relatively constant with depth and the abundance of these cells does not decline in the mixed layer (Olson et al. 1990). In contrast, in the subtropical Pacific L_1 is

always present in excess of the total copper concentration so the free Cu^{2+} is low at 0.01 to 0.2 pM (Coale and Bruland 1990). In this environment, *Prochlorococcus* are two orders of magnitude more abundant than *Synechococcus* at the surface throughout the year (Campbell and Vaulot 1993, Campbell and Nolla 1994).

To test the hypothesis that free Cu^{2+} may play a role in regulating the relative abundance of *Prochlorococcus* and *Synechococcus* in the Sargasso Sea two approaches were used. First, the sensitivity of cultured isolates to environmentally relevant free Cu^{2+} concentrations was determined in the laboratory using *Prochlorococcus* isolates representing both of the major ecotypes (determined by chl B/A ratios (Moore et al. 1998, Moore and Chisholm 1999)) and *Synechococcus* clones isolated from the Sargasso Sea. Second, the copper sensitivity of *in situ* populations of cyanobacteria in environments with a range of free Cu^{2+} was compared by taking water from different seasons and from different depths in stratified water columns. The sensitivity to copper was determined by following changes in cell number (the net growth rate), pigment fluorescence, and DNA content during on deck bottle incubations.

METHODS

Culture experiments.

Seawater for the culture media was collected from the Sargasso Sea and stored in acid cleaned polyethylene carboys. The water was sterilized by micowaving after filtration through a 0.2μ filter (Keller et al. 1988) using an acid washed borosilicate glass filter flask. Nutrient and trace metal stocks were sterilized separately by filtration through acid cleaned 0.02 or 0.2μ syringe filters. The composition of the media is listed in Table 1. Metals were buffered by 10^{-4} M nitrilotriacetic acid (NTA). Copper was added as $CuCl_2$ at 10, 32, 65, 320, and 3200 nM and free Cu^{2+} calculated as in Brand (1986) were 1.0, 1.5, 3.0, 15, and 150 pM.

All experimental manipulations were done in a laminar flow hood and sterile technique was maintained but none of the *Synechococcus* and *Prochlorococcus* isolates used in this experiment (Table 2) were axenic. Marine bacteria can produce copper binding compounds (Schreiber et al. 1990), but copper toxicity

Table 1) Media composition.

Media Component	Total concentration
Nitrilotriacetic acid (NTA)	10^{-4} M
NH_4Cl	50 μM
Urea	100 μM
$NaH_2PO_4\ H_2O$	10 μM
Na_2CO_3	40 μM
$FeCl_3\ 6H_2O$	7.3 μM
$Na_2MoO_4\ 2\ H_2O$	0.003 μM
$MnCl_2\ 4H_2O$	0.45 μM
$CoCl_2\ 6H_2O$	0.16 μM
$NiCl_2\ 6\ H_2O$	0.13 μM
Na_2SeO_3	0.01 μM
$ZnSO_4\ 7H_2O$	0.32 μM

was not affected by the presence of bacteria in an EDTA buffered media (Appendix B). The *Synechococcus* isolates used, WH7805 and WH8103, were both isolated from the Sargasso Sea and have pigment compositions characteristic of open ocean strains (Waterbury et al. 1986). *Prochlorococcus* isolates were from a variety of locations and represented both the high and low chl B/A ecotypes (Moore et al. 1998, Moore and Chisholm 1999). Cyanobacteria were grown in acid washed borosilicate glass tubes in a 14:10 light dark cycle at an irradiance of 30 $\mu Em^{-2}sec^{-1}$. Cells were grown in continuous batch culture for three or more transfers at constant cell division rates before the experiment was started. Data was collected from at

Table 2) Isolation information and characteristics of *Synechococcus*

and *Prochlorococcus* isolates used in this study.

Isolate Name	Isolation coordinates
Synechococcus:	
WH7805	33° 44.8' N; 67° 30' W
WH8103	28° 30' N; 67° 23.5' W
Prochlorococcus:	
	High chl B/A ecotype, low light adapted
9313	37° 30.8'N; 68° 14.4'W
SS120	28° 59'N; 64° 21'W
	Low chl B/A ecotype, high light adapted
Med 4	43° 12'N; 6° 52'W
9311	37° 30.8'N: 68° 14.4'W
9401	Sargasso Sea

For more information on the *Synechococcus* and *Prochlorococcus* isolates used see

(Waterbury et al. 1986) and (Partensky et al. 1993, Moore et al. 1995, Moore 1997,

Moore et al. 1998, Moore and Chisholm 1999), respectively.

least two transfers after the addition of copper. In vivo fluorescence was measured

using a Turner fluorometer and cell division rates were determined using this

parameter as a proxy for cell number. Cell division rates based on changes in

fluorescence are accurate only if cells are in balanced growth (Brand et al. 1986).

Because copper can influence the chlorophyll content per cell, cell division rates

were set at zero when increases in fluorescence could not be maintained at a constant

rate (an indication that cells were not in balanced growth).

Field experiments.

Station descriptions. Three stations were sampled in order to determine the copper

sensitivity of cyanobacteria from different environments. The location (Fig 1),

sampled depths and characteristics of each station, including the free Cu^{2+}

concentrations in the control bottles, are summarized in Table 3 and described

below.

In the winter in the northern Sargasso Sea the water column was well mixed, with a

mixed layer depth of approximately 200m (Fig 2A). There was a small break in

temperature close to 100m that is not evident in the graph (Fig 2A). In this

environment free Cu^{2+} concentrations are expected to be low throughout the water
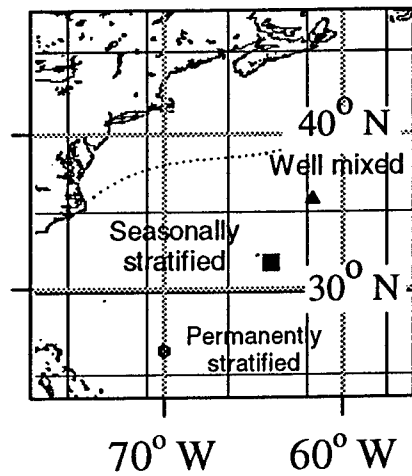
Fig 1) Map of stations sampled in the Sargasso Sea. Different symbols represent different cruises. The January 1997 station was well mixed, the June 1998 station was seasonally stratified and the February 1998 station was permanently stratified.
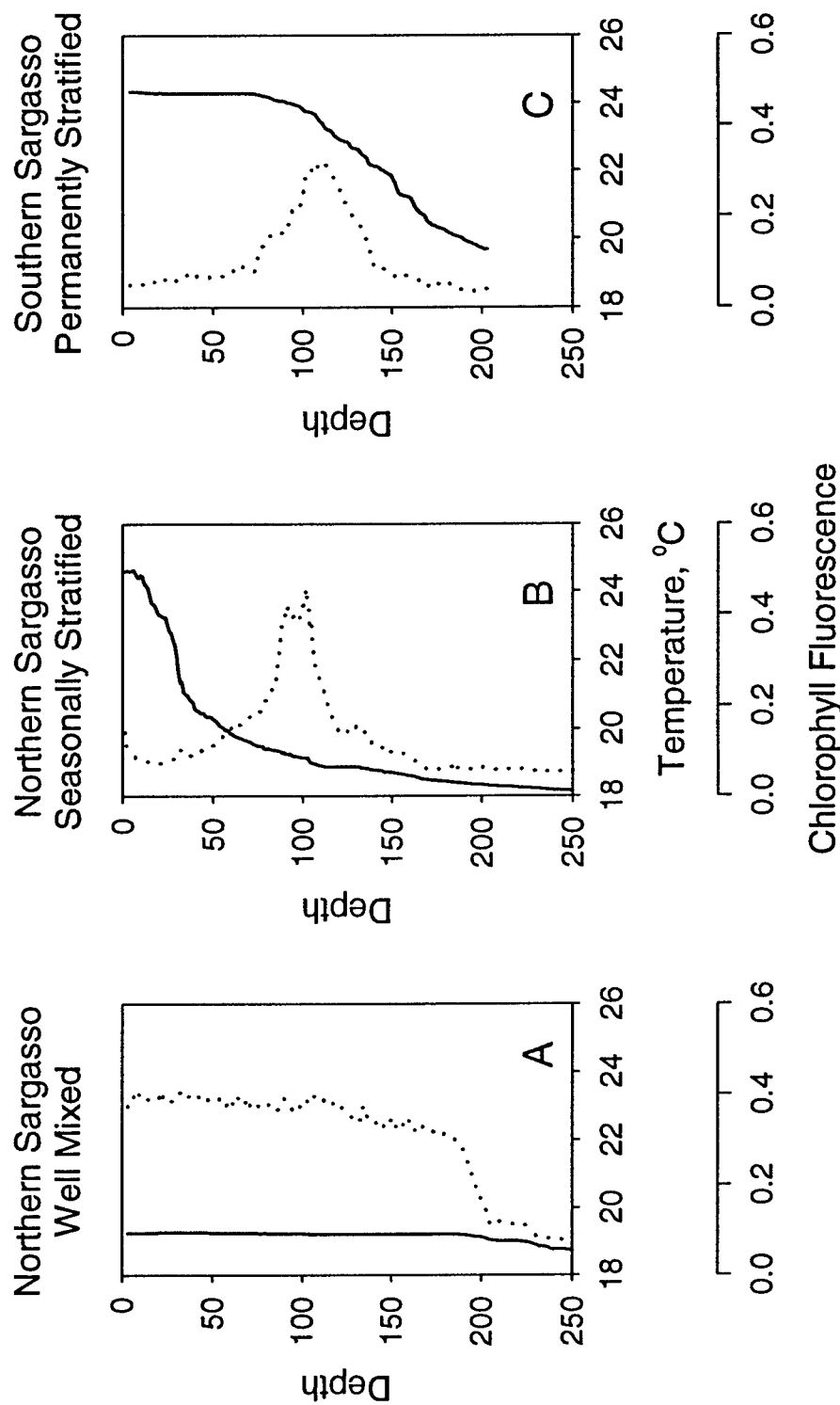
Fig 2) Depth profiles of temperature (solid line) and relative chlorophyll fluorescence (dotted line) for **A)** the well mixed and **B)** seasonally stratified water columns in the northern Sargasso Sea. **C)** Data from the permanently stratified water column in the southern Sargasso. Note that the chlorophyll fluorescence scales are relative and not directly comparable in A, B, and C.

Table 3) Summary of station characteristics and conditions in the bottles incubated on-deck. Bottles were screened with optical gels to attain the percent of incident irradiation values (%Io) listed. The free Cu^{2+} concentrations in control bottles were determined by J. W. Moffett using adsorptive cathodic stripping voltammetry with benzoylacetone as the competing ligand (Moffett 1995). Values marked with an (*) were not determined directly, but were estimated from previous results (Moffett et al. 1990, Moffett 1995).

Date	Location	Description	Depth Sampled	% Io	Free Cu^{2+}
January 1997	Northern Sargasso	Seasonally well mixed 200m mixed layer	45m	20%	0.6 pM
June 1998	Northern Sargasso	Seasonally stratified: 25m mixed layer	16m 85m	9% 2.3%	3.6 pM 0.1 pM
February 1998	Southern Sargasso	Permanently stratified: 75m mixed layer	50m 125m	9% 2.3%	2 pM <0.5 pM *

column (Moffett 1995) and were less than 1 pM in the control bottle from 45m (Table 3). Two stratified water columns were also investigated, one in the northern Sargasso Sea and a second further south. There are several important differences between these sites including the extent and duration of stratification. Stratification of the northern Sargasso Sea is seasonal and occurs only in the summer. In June there was a shallow 25m mixed layer, with some additional stratification between 10 and 12m (Fig 2B). The water column in the southern Sargasso Sea is permanently stratified and in February had a mixed layer of 75m (Fig 2C). In both water columns, free Cu^{2+} concentrations were high at the surface and low below the mixed

layer. This pattern was observed both in the control bottles and in previous depth profiles of free Cu^{2+} *in situ* (Moffett et al. 1990, Moffett 1995, Table 3). These stations will be referred to as well mixed, seasonally stratified and permanently stratified, respectively.

Bottle incubations. The copper sensitivity of cells in environments with a range of *in situ* free Cu^{2+} was compared by taking water from different seasons and from different depths in stratified water columns and adding copper. Water for these incubations was taken from 45m in the well mixed station and from both within and below the mixed layer in the stratified water columns (Table 3). In each case 0, 2, and 5 nM total copper was added to duplicate or triplicate bottles. The free Cu^{2+} resulting from some of these copper additions are listed in Table 4.

Seawater was collected using acid cleaned, Teflon coated Go-Flo bottles suspended on Kevlar wire. Further experimental manipulations were done under a laminar flow hood. Samples from the on-deck incubation bottles were taken through acid cleaned Teflon transfer caps and tubing via a positive pressure system. This allowed trace metal clean sampling without moving the bottles into the laboratory. Incubation bottles were kept cool with surface seawater and were screened using optical gels to mimic the *in situ* illumination (Table 3, Appendix B).

Sample preservation and DNA staining with Sybr Green 1. All samples taken from the bottles were preserved in a final concentration of 0.1% glutaraldehyde for 10 minutes at room temperature in the dark and were then stored in liquid nitrogen (Vaulot et al. 1989). Selected samples were also stained for DNA by adding potassium citrate (final concentration 30 μM) and a 1:10^{-4} final dilution of Sybr Green 1 (Molecular Probes). Samples were maintained at room temperature in the dark for 15 minutes before analyzing by flow cytometry. All working stocks of Sybr Green I were diluted in DMSO (Marie et al. 1997). In order to determine the fraction of cells in S and G_2+M, DNA fluorescence histograms were modeled and divided into G_1, S, and G_2+M using ModFit software (Verity Software House, Inc.).

Flow cytometry. Cell number and the amount of DNA, chlorophyll fluorescence, and FALS per cell (an indication of cell size) were quantified using a modified EPICS 753 flow cytometer. The instrument was setup as described in Binder et al. (1996) except it was used in single beam mode and the optical filters were arranged to detect green fluorescence from Sybr Green I, which is excited at 488 nm. After passing through 630 and 560 nm short-pass dichroic filters, green signals passed though a 515 nm long-pass filter. Samples were delivered at a constant rate using a syringe pump (Harvard Apparatus, model 22). Unstained samples were run at a flow rate of 10 μl min^{-1}. Sybr Green stained samples were run at lower flow rates (5 to 7

µl min^{-1}) after pre-equilibrating sample lines with the stain for at least one hour to achieve the lowest coefficients of variation for the DNA histograms.

Determination of net growth rates. Net growth rates (k day^{-1}) were determined from the change in cell number over time using

$$k = \frac{\ln N_t - \ln N_{t_0}}{t - t_0} \quad (1)$$

Where N_t and N_{t_0} are the cell numbers at time t (the beginning of the bottle incubation) and t_0 (the end of the incubation, in days). Regression coefficients are reported ± the standard error in Table 4.

RESULTS and DISCUSSION

Free cupric ion concentrations and cell division rates in culture.

The copper sensitivity of marine cyanobacteria was analyzed by measuring the cell division rates of cultured isolates at a range of free Cu^{2+}. *Synechococcus* cell division rates were not affected by free Cu^{2+} up to 150 pM (Fig 3). These clones were chosen because they were among the most copper sensitive *Synechococcus*

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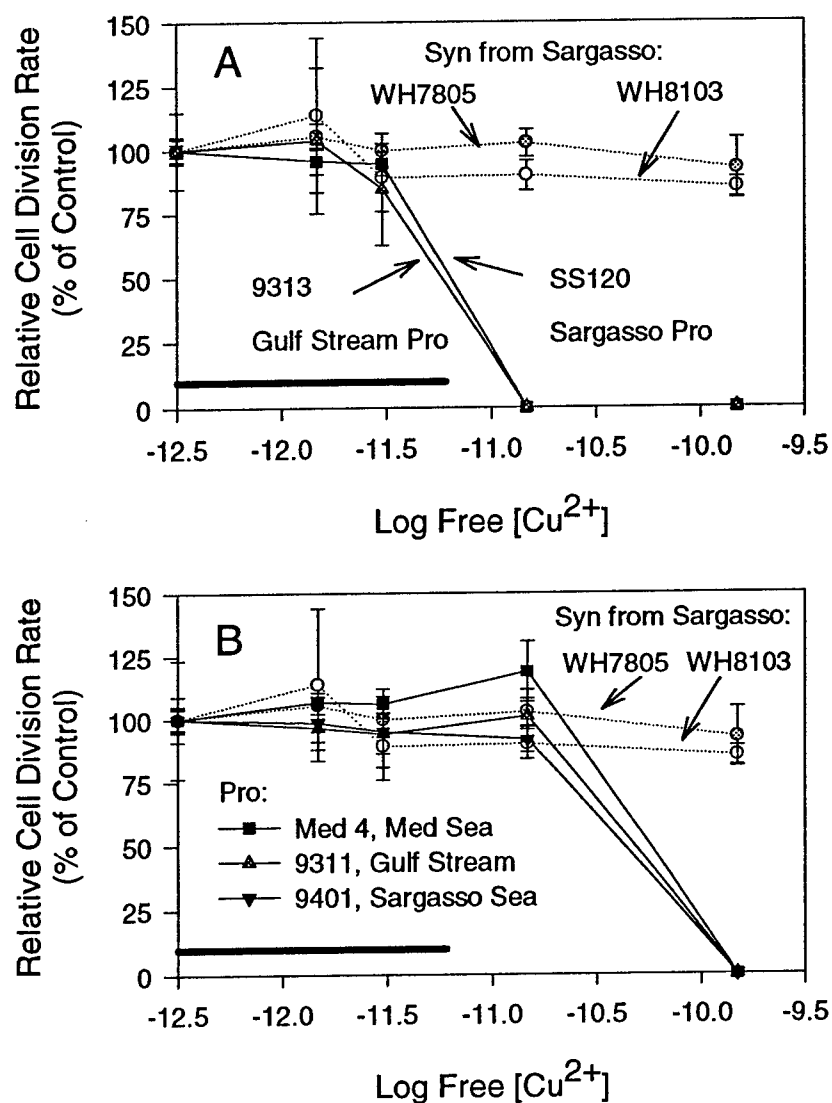
Fig 3) Cell division rates of *Prochlorococcus* and *Synechococcus* isolates as a function of free Cu^{2+}. The cell division rates are relative to those of the control. The average cell division rate of the controls was 0.6 day^{-1}, with a range of 0.35 to 0.86 day^{-1}. The dark bar represents the range of free Cu^{2+} expected in the Sargasso Sea. **A)** Isolates from the Sargasso Sea and Gulf Stream. The *Prochlorococcus* isolates are all high chl B/A ecotypes (low light adapted, Table 2). **B)** Isolates from the Sargasso Sea, the Gulf Stream, and the Mediterranean Sea. The *Prochlorococcus* isolates are all low chl B/A ecotypes (high light adapted, Table 2).

isolates in an earlier study (WH7805, Brand et al. 1986) or have a pigment composition that is characteristic of the dominant type of *Synechococcus* in the Sargasso Sea (WH8103, Olson et al. 1988).

The *Prochlorococcus* isolates were more copper sensitive than *Synechococcus*, none could grow at the highest copper concentration where *Synechococcus* was able to maintain maximum cell division rates (Fig 3). However, *Prochlorococcus* with a high chl B/A ratio (low light adapted, Moore et al. 1998, Moore and Chisholm 1999) were more sensitive to copper than high light adapted isolates with a low chl B/A ratio (compare Fig 3A and 3B) (Moore et al. 1998, Moore and Chisholm 1999). The two high chl B/A strains, SS120 and 9313, could not grow at free Cu^{2+} above 3 pM. With SS120 this was the case even if the cells were acclimated to lower levels of copper before exposure to higher free Cu^{2+} concentrations. The low chl B/A strains, Med 4, 9311 and 9401, were more copper resistant and cell division was reduced only at 150 pM. Linkage of a "high light adapted" pigment composition and copper resistance is not surprising since free Cu^{2+} and irradiance are both high in shallow mixed layers (Moffett 1995).

We might expect that *Prochlorococcus* is more likely to be influenced by the free Cu^{2+} in the Sargasso Sea than *Synechococcus*, since the cell division rate of at least some *Prochlorococcus* strains was significantly reduced at free Cu^{2+} that are close to

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those found *in situ*. Within the *Prochlorococcus* population the relatively copper

resistant cells would be expected to dominate in environments where both irradiance

and free Cu^{2+} are high. In other words, one way that copper could influence

cyanobacteria ecology is by exerting a selective pressure for cells that can tolerate

high free Cu^{2+}.

However, applying results from culture data to the field is considerably complicated

by the fact that trace metal toxicity is dependent on the ratio of toxic to nutrient

metals. These competitive interactions in which a high concentration of one metal

alleviates the toxic effects of another have been found for Cu and Fe (Murphy et al.

1984), Cu and Mn (Sunda 1986, Sunda and Huntsman 1998a, Sunda and Huntsman

1998b) as well as Cu and Zn (Sunda and Huntsman 1998a). For example, both

Synechococcus and *Prochlorococcus* are more copper sensitive in a media

containing lower concentrations of other trace metals (Brand et al. 1986, Appendix

B). However, the relative sensitivity of cyanobacteria to copper was maintained and

Prochlorococcus were still more sensitive to copper than *Synechococcus* (Appendix

B).

Copper sensitivity of cyanobacteria in the field.

Well mixed water columns.

The copper sensitivity of cyanobacteria from environments with different *in situ* free Cu^{2+} concentrations was analyzed by incubating natural phytoplankton assemblages from different seasons and different depths with added copper. The northern Sargasso Sea in the winter is one example of a low copper environment (Tables 3 and 4). Water from 45m in this well mixed water column was incubated on deck with 0, 2, or 5 nM of added total copper. Measured total soluble copper in the control bottles was 1.2 nM. Greater than 99% of this copper was complexed with organic ligands and as a result the free Cu^{2+} was much lower at 0.6 pM (Tables 3 and 4). This is in agreement with previous results of *in situ* free Cu^{2+} concentrations of less than 0.5 pM throughout a similar water column (Moffett 1995). Free Cu^{2+} in the + Cu bottles increased to 4.7 and 9.0 pM with the addition of 2 and 5 nM copper, respectively (Table 4). For comparison, the highest *in situ* free Cu^{2+} reported for the Sargasso Sea is 6 pM. This free Cu^{2+} concentration was measured in a shallow mixed layer in the seasonally stratified northern Sargasso Sea (Moffett 1995).

Copper had a dramatic effect on the *Prochlorococcus* population. By day 4 only one out of the triplicate 5 nM Cu incubation bottles had an identifiable *Prochlorococcus*
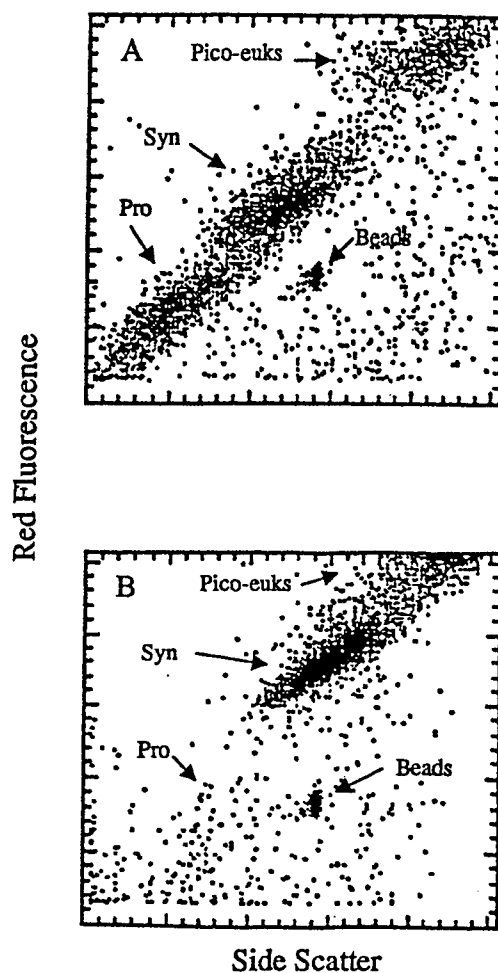
Fig 4) Side scatter versus chlorophyll fluorescence relative to standard beads in A) the control and B) a 5 nM added copper bottle, both 4 days after copper was added. Water for these on deck incubations was taken from the well mixed water column at 45m in the northern Sargasso Sea. Populations of pico-eukaryotes, *Synechococcus*, *Prochlorococcus* and 0.474μm standard beads are identified. Note that *Prochlorococcus* were virtually eliminated by the addition of copper but the other phytoplankton were not.
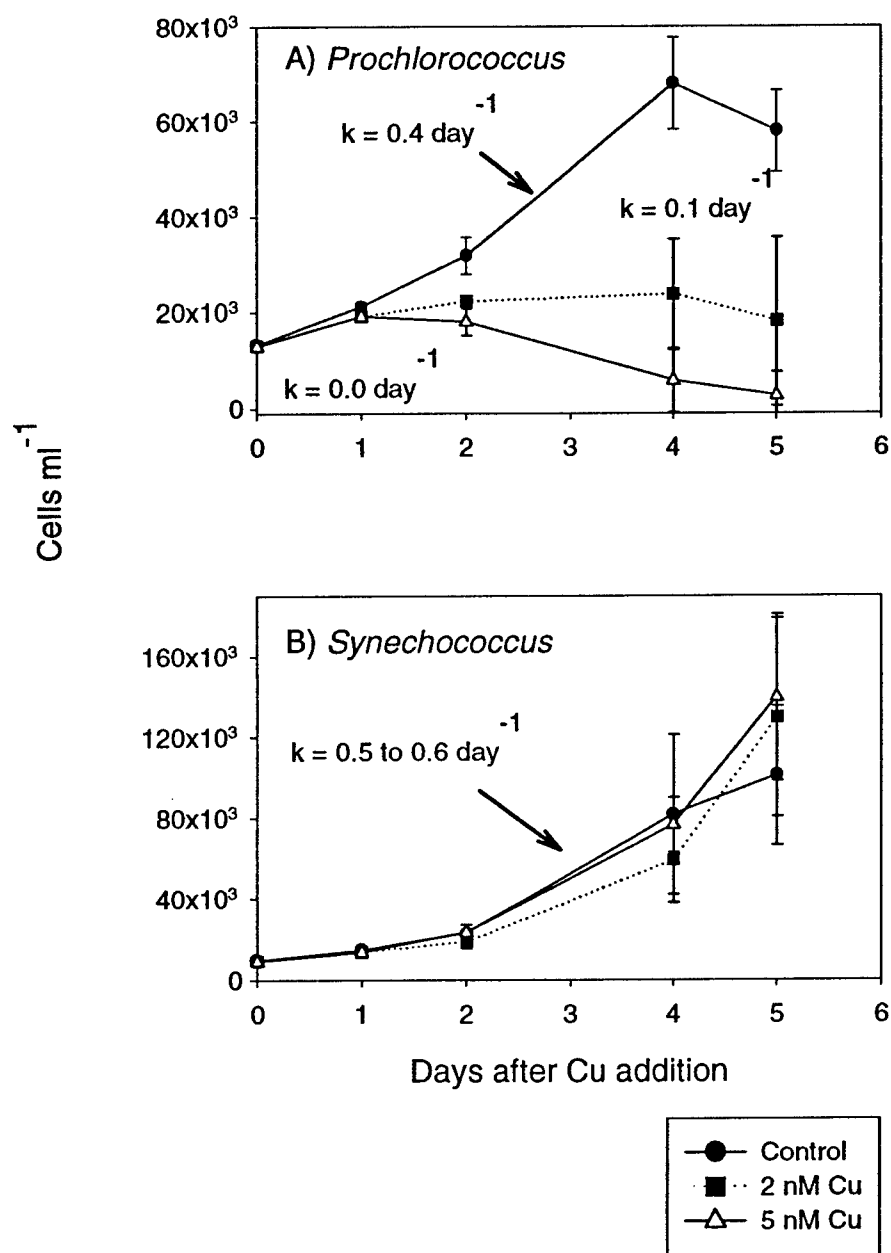
Fig 5A) *Prochlorococcus* and **B)** *Synechococcus* abundance and net growth rates in triplicate bottles incubated on deck with 0, 2, and 5 nM added copper. Free Cu^{2+} concentrations were 0.6, 4.7, and 9.0 pM, respectively (see Table 4). Water for these incubations was taken from 45m in a well mixed water column (January 1997). The net growth rates were calculated over the first four days.

population (Fig 4). Net growth rates (calculated using equation 1) were significantly depressed and decreased from 0.4 day^{-1} to 0.1 and 0.0 day^{-1} with the addition of 0, 2 and 5 nM Cu, respectively (Fig 5A and Table 4). *Synechococcus* was less sensitive to copper than *Prochlorococcus* and net growth rates ranged from 0.5 to 0.6 day^{-1} in both the control and copper bottles (Fig 5B and Table 4).

Stratified water columns.

The free Cu^{2+} concentration in stratified water columns varies significantly with depth and can be an order of magnitude higher in shallow mixed layers than below the thermocline (Moffett 1995). For instance, in the seasonally stratified water column measurements of free Cu^{2+} in the control bottles were 36 fold higher in the ML compared to deeper water (Table 4), which is in agreement with previous results (Moffett 1995). In the stratified water columns on-deck incubations of cyanobacteria with added copper were completed both within and below the mixed layer (ML and BML, respectively) in order to compare the copper sensitivity of cyanobacteria from environments with different *in situ* free Cu^{2+} concentrations. The free Cu^{2+} concentrations resulting from the addition of 0, 2, or 5 nM total added copper are listed in Table 4. The addition of 2 nM copper generally resulted in free Cu^{2+} concentrations that are representative of those seen *in situ*, while the 5 nM copper addition increased free Cu^{2+} over environmentally relevant levels. (The free Cu^{2+} in

the + Cu bottles from the permanently stratified water column have not yet been

determined.)

Table 4) *Prochlorococcus* and *Synechococcus* net growth rates (k day^{-1}) with total added copper and the resulting free Cu^{2+} concentrations during on-deck bottle incubations. Free Cu^{2+} in these bottles were determined by J. W. Moffett using adsorptive cathodic stripping voltammetry with benzoylacetone as the competing ligand (Moffett 1995). Values marked with an (*) were not determined directly, but were estimated from previous results (Moffett et al. 1990, Moffett 1995). Net growth rates ± standard errors are reported. Those that are significantly different (99% confidence levels using an F-test to compare slopes) from the control are in bold. ND = not determined.

Description	Depth Sampled	Total Cu, nM	Free Cu^{2+} pM	*Prochlorococcus* k day^{-1}	*Synechococcus* k day^{-1}
Well mixed:	45m	0	0.6	0.4 ± 0.02	0.5 ± 0.10
200m mixed layer		2	4.7	**0.1 ± 0.05**	0.5 ± 0.04
		5	9.0	**0.0 ± 0.01**	0.6 ± 0.03
Seasonally stratified:					
25m mixed layer	16m	0	3.6	-0.1 ± 0.03	0.0 ± 0.02
		5	>30*	-0.2 ± 0.11	**-0.1 ± 0.02**
	85m	0	0.1	0.1 ± 0.05	-0.0 ± 0.05
		2	5.0	**-0.1 ± 0.04**	-0.2 ± 0.16
		5	31.6	**-0.5 ± 0.16**	-0.2 ± .11
Permanently stratified:					
75m mixed layer	50m	0	2	0.1 ± 0.02	0.1 ± 0.02
		2	ND	**-0.0 ± 0.04**	0.1 ± 0.03
		5	ND	**-0.1 ±0.03**	0.1 ±0.04
	125m	0	<0.5 *	0.1 ± 0.03	0.3 ± 0.04
		5	ND	**-1.2 ± 0.23**	0.2 ± 0.06

The *in situ* concentration of free Cu^{2+} had a strong effect on the copper sensitivity of

Prochlorococcus. Cells from the ML, where the *in situ* free Cu^{2+} was already high,

were much less sensitive to copper than those in deeper water. Copper had no

Prochlorococcus
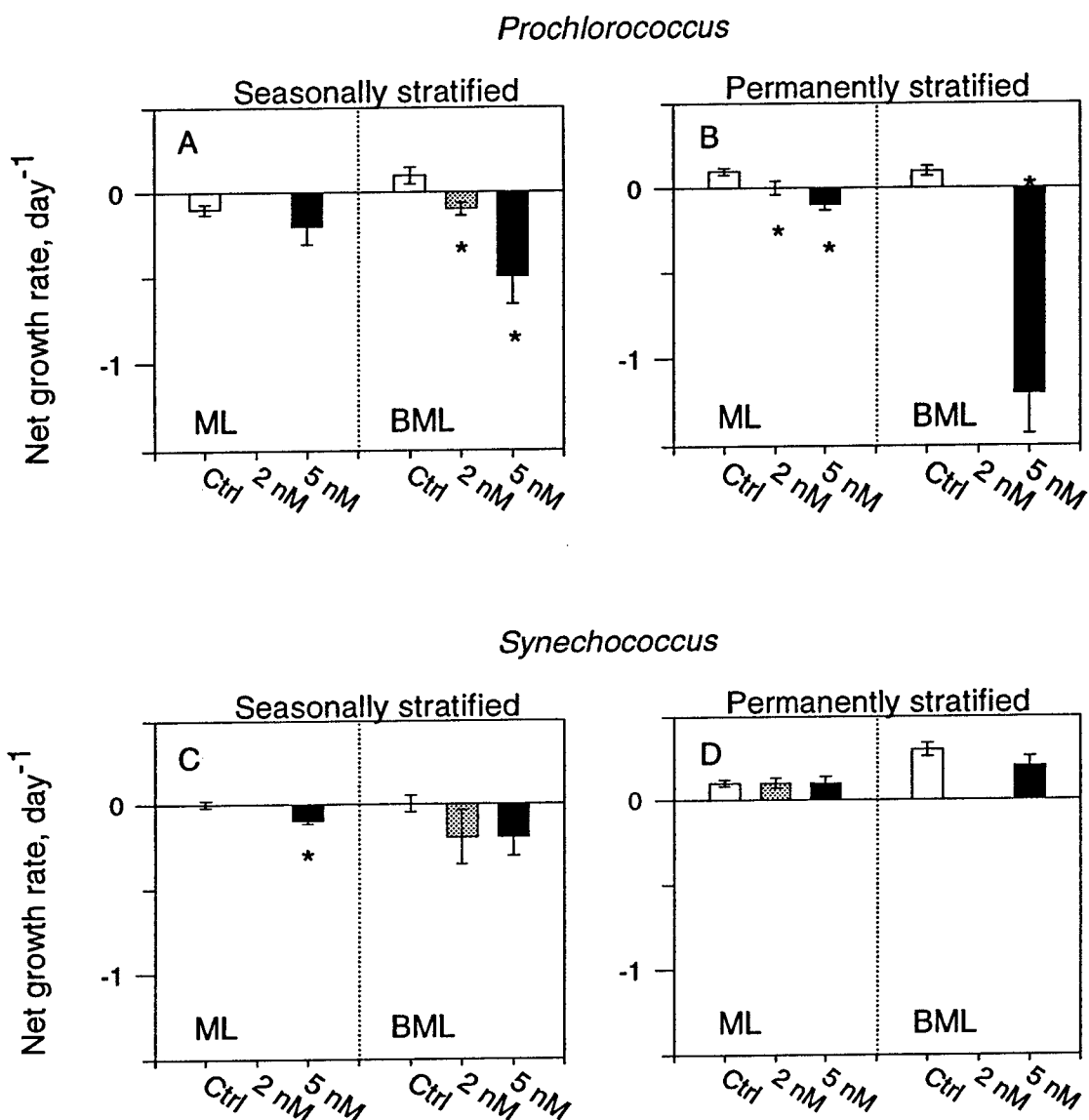
Synechococcus

Fig 6) *Prochlorococcus* and *Synechococcus* net growth rates in bottles incubated with 0, 2, and 5 nM of added copper. For free Cu^{2+} concentrations see Table 4. **A** and **C)** *Prochlorococcus* and *Synechococcus* from the seasonally and **B** and **D)** permanently stratified water columns. ML and BML refer to incubations of water from the mixed layer and below the mixed layer, respectively. Error bars represent the standard errors of the net growth rate regressions and significant differences between the net growth rates in the control and +Cu bottles are marked with an *.

significant effect on the net growth rate of *Prochlorococcus* from the seasonal mixed layer, although the trend was for lower cell numbers in the + Cu bottle, even though the free Cu^{2+} in the 5 nM added Cu bottle was probably over 30 pM (Fig 6A, Table 4). This is about 5x the highest *in situ* concentration seen in the Sargasso Sea. In contrast, most of the *Prochlorococcus* from BML were extremely sensitive to copper. In the seasonally stratified station, net growth rates decreased from 0.1 ± 0.05, to -0.1 ± 0.04, and –0.5 ± 0.16 day^{-1} for the control, 2 and 5 nM copper bottles, respectively (Fig 6A and Table 4). A similar pattern was seen in the permanently stratified mixed layer. The net growth rates of *Prochlorococcus* from the mixed layer did decrease significantly, from 0.1 ± 0.02 to –0.1 ± 0.03 in the control and 5 nM added Cu bottles, respectively. However, BML the loss of cell numbers when 5 nM Cu was added was even greater and the net growth rate decreased to −1.2 ± 0.23 day^{-1} (Fig 6B, Table 4).

The *in situ* concentration of free Cu^{2+} did not have a strong effect on the copper sensitivity of *Synechococcus*, which varied with depth to a much lesser extent than for *Prochlorococcus*. The most significant decrease in *Synechococcus* net growth rate occurred in the ML of the seasonally stratified water column where the *in situ* free Cu^{2+} was already high (Fig 6D and Table 4). Below the mixed layer, where the *in situ* free Cu^{2+} was low, the addition of copper to water from both the seasonally

and permanently stratified water columns made no significant difference in the net growth rates of *Synechococcus*.

Synechococcus were significantly more copper resistant than *Prochlorococcus* from below the mixed layer, which were in general extremely copper sensitive. However, a small fraction of the *Prochlorococcus* population in deep water appeared to be as resistant to copper as *Synechococcus*. The chlorophyll fluorescence of the majority of the *Prochlorococcus* from BML in the seasonally stratified water column dropped significantly after exposure to elevated copper concentrations (Fig 7). It is the loss of these cells that caused the large decreases in cell number in the + Cu bottles. In a small number of "unaffected" cells the chlorophyll fluorescence did not decrease when copper was added (Fig 7).

In addition, an analysis of *Prochlorococcus* DNA histograms from control and 5 nM Cu bottles indicated that the "unaffected cells" were undergoing cell division while the "affected cells" were not. In the seasonally stratified water column, DNA histograms from the control and 5 nM Cu bottles were compared on day 3.5 at 22:30 (Fig 8), when cells were expected to be in S or G_2+M (Vaulot et al. 1995, Liu et al. 1997, Shalapyonok et al. 1998). Cells from the control and the "unaffected cells" in the 5 nM Cu bottle had similar DNA histograms, with 31 and 45% of the cells in the S phase, respectively. These *Prochlorococcus* were not simply arrested in these
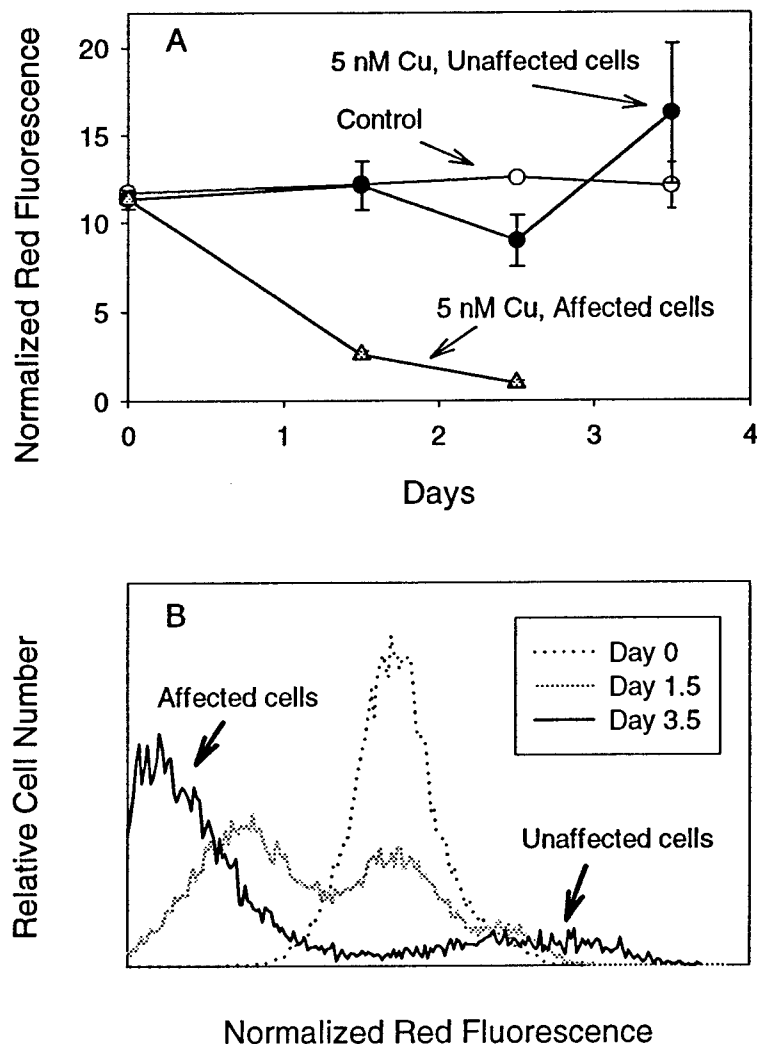
Fig 7A) *Prochlorococcus* red fluorescence per cell normalized to standard beads
with time in the control and 5 nM Cu bottles. The *Prochlorococcus* population
split into cells that rapidly lost chlorophyll fluorescence and cells unaffected by the
addition of copper. Water for this incubation was taken from below the mixed
layer in the seasonally stratifed water column. **B)** Normalized red fluorescence
histograms from the 5 nM copper bottle on days 0, 1.5 and 3.5 from the same
experiment. Most of the cells were affected by the addition of copper within 1.5 days
and started to decrease in fluorescence. A small subpopulation of unaffected cells
with high red fluorescence remained even after 3.5 days. Data from the cells
affected by copper on day 3.5 is not graphed in Fig 7A because this population is
so dim it could not be entirely resolved from the noise.
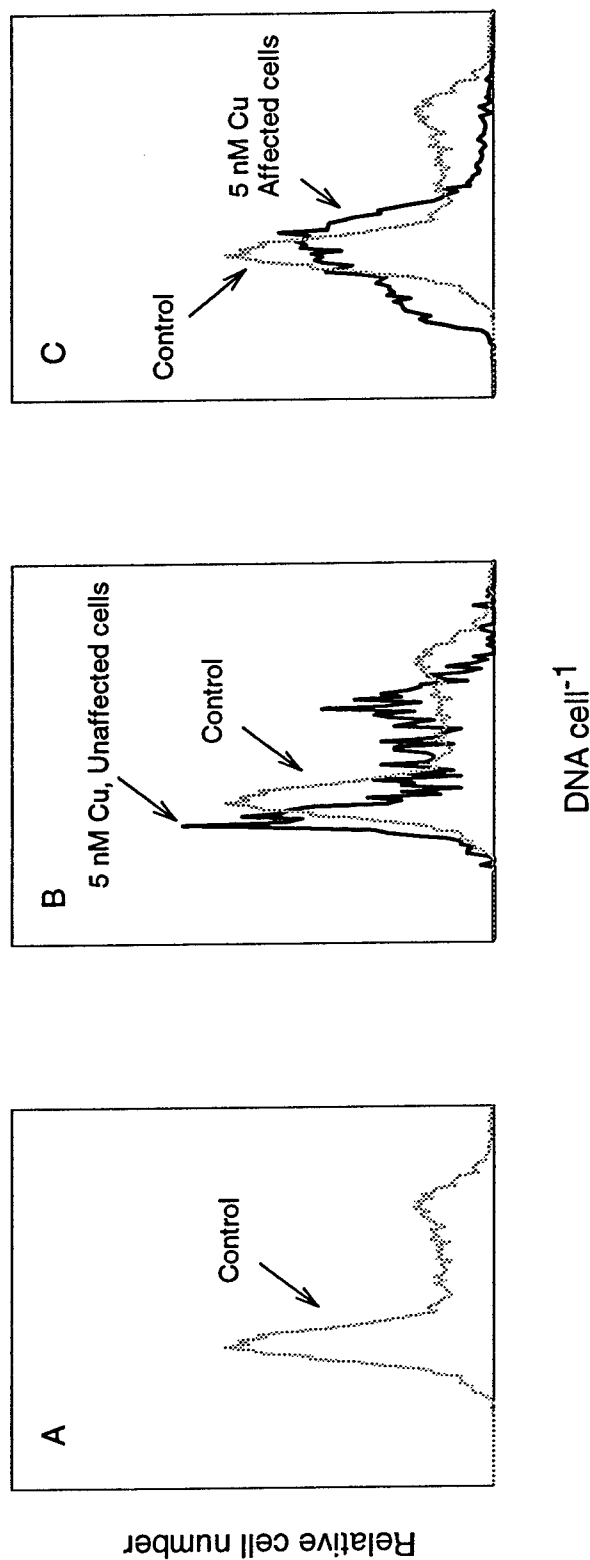
Fig 8) DNA histograms from **A)** the control, **B)** *Prochlorococcus* unaffected and **C)** affected by the addition of copper in the 5 nM Cu bottle. Data from the control is repeated in all of the graphs. Samples were taken during on deck bottle incubations at 22:30. Water for this incubation was taken from below the mixed layer in the seasonally stratified water column. The DNA histograms were normalized to standard beads and to the total number of events. The unaffected cells in B were about 10% of the total population. This subpopulation of cells contains slightly less DNA per cell than seen in the control. The affected cells in C are arrested in G_1 and early S (see text). Note that this G_1 peak has a left shoulder representing cells with degraded DNA.

stages of the cell cycle since the percent of cells in S earlier in the day was only 13 to

14% (data not shown). The greatest difference between the two DNA histograms is

that the DNA content of the "unaffected cells" is slightly less than that of the control

(Fig 8). Occasionally two peaks of cells in G_1 were also seen in *in situ* DNA

histograms from BML (data not shown). This may be an indication that two

Prochlorococcus populations with different DNA contents were present, similar

results have been reported in the subtropical Pacific (Campbell and Vaulot 1993).

In contrast, cells "affected" by the addition of 5 nM copper were arrested in G_1 and

early S (Fig 8). Few to no cells in G_2+M were present and the DNA histograms did

not change with time, indicating that cell division was not occurring (data not

shown). There was also a significant left shoulder to the G_1 peak, which reflects the

degradation of DNA in dead and dying cells. A similar pattern of arrest in G_1 and

early S with substantial DNA degredation after the addition of copper was also seen

in axenic cultures of the *Prochlorococcus* isolate Med 4 as well as in the

permanently stratified water column below the mixed layer (Appendix B).

Net growth rate, grazing and cell division rates.

Net growth rates are a function of both the cell division rate and mortality and can

therefore be influenced by changes in grazing rate. However, it is unlikely that

differences in grazing were a significant factor in these experiments. For one, we have shown that progression through the cell cycle was modified by copper, which implies that in at least some *Prochlorococcus* actual cell division rates were affected in the + Cu bottles (Fig 8). In addition, in environments where the free Cu^{2+} was low *Prochlorococcus* numbers decreased significantly while the *Synechococcus* population was relatively unaffected. This was especially true for the well mixed water column where *Synechococcus* were increasing exponentially while *Prochlorococcus* were essentially disappearing (Fig 5). To account for these contrasting patterns, grazing would have to be extremely selective in favor of *Prochlorococcus*. In addition, cell numbers decreased in + Cu bottles. As a result, in order for changes in grazing rate to play a significant role in reducing the net growth rates, the grazing rate on *Prochlorococcus* must be higher in the + Cu bottles than in the controls. Cell division rates of ciliates in culture are reduced by free Cu^{2+} of less than a 1 pM (Stoecker et al. 1986), but it is unlikely that copper would selectively inhibit grazers specific for *Synechococcus*.

Resistance to copper toxicity.

Prochlorococcus were sensitive to copper in the well mixed water column and BML in the stratified water columns, environments where the free Cu^{2+} was low (Moffett et al. 1990, Moffett 1995). *Prochlorococcus* were less sensitive to copper toxicity in

mixed layers where the free Cu^{2+} is usually high. One explanation for these

differences is that high copper environments selected for copper resistant strains.

We hypothesize that the copper resistant cells in the field were members of the low

chl B/A ecotype (Moore et al. 1998) since clones in this group were copper resistant

in the laboratory (Fig 3). In the northern Sargasso this would lead to a seasonal

succession of *Prochlorococcus* ecotypes with copper resistant, low chl B/A cells

dominating in the mixed layers during the summer.

Prochlorococcus copper sensitivity could also vary with depth and season if copper

resistance mechanisms within a genetically identical population were induced only

when free Cu^{2+} was high. This was probably the case to some extent. For instance,

the production of the copper binding L_1 by *Synechococcus* increases when free Cu^{2+}

is high (Moffett and Brand 1996). However, there are several lines of evidence to

support the conclusion that at least some of variations in copper sensitivity were due

to the dominance of different ecotypes. For one, stepwise acclimation to increasing

concentrations of copper, which would allow for the induction of copper resistance

mechanisms, had no effect on the copper sensitivity of *Prochlorococcus* isolate

SS120 (data not shown). More importantly, *in situ* populations of *Prochlorococcus*

are genetically diverse (Urbach and Chisholm 1998), genetically distinct ecotypes

have been shown to coexist in the field (Moore et al. 1998) and to date all members

of the low chl B/A ecotype have also been relatively copper resistant (Fig 3, Table

2). Therefore, differences in copper tolerance in the field may have a genetic basis as well. This is made more likely by the fact that a small proportion of the *Prochlorococcus* population below the mixed layer was relatively unaffected by copper (Figs 7 and 8).

The molecular basis for the differences in copper sensitivity between *Prochlorococcus* ecotypes is unknown. Strategies used by bacteria to gain copper tolerance include copper efflux mechanisms such as Cu ATPases, sequestration by periplasmic or outer membrance proteins, adsorption onto polysaccharides on the cell surface and reduced copper transport (Cooksey 1993, Brown et al. 1994, Cervantes and Gutierrez-Corona 1994) as well as production of copper chelators (Moffett and Brand 1996). Some genes responsible for copper tolerance in bacteria are located on plasmids which could be transferred between bacteria. Other copper regulation genes, especially those dealing with normal copper metabolism, are chromosomal (Brown et al. 1994).

Whatever the mechanism of copper tolerance in *Prochlorococcus* it seems to come at a price. *Prochlorococcus* numbers in the mixed layer in the seasonally stratified water column are very low, in spite of the fact the net growth rates of these cells were not significantly affected by copper. One possible explanation is that the mechanism of copper tolerance is energy intensive and the cell division rates of

Prochlorococcus in the mixed layer are low as a result (an attempt to measure the cell division rate directly was inconclusive, see Chapter 4). ATPases for instance can consume a significant amount of energy. It has been estimated that the Na+-K+ ATPase in mammalian cells accounts for 30% of the total energy budget (Lippard and Berg 1994). Copper tolerance may not confer an adaptive advantage in cells that are adapted to low light (high chl B/A ecotype). *Prochlorococcus* at depth have higher pigment concentrations and therefore have a higher requirement for other trace metals such as Fe and Mn. Mechanisms designed to increase the cell quotas of these metals are therefore necessary, even if they inadvertently increase the Cu concentration per cell as well (Sunda and Huntsman 1997, Sunda and Huntsman 1998b).

The small *Prochlorococcus* subpopulation below the mixed layer that was unaffected by copper (Figs 7 and 8) also indicates that the variations in copper sensitivity were due more to intrinsic differences in the cells than to physical or chemical factors. However, differences in irradiance and the trace metal concentrations between the surface and deeper water may also influence the degree of copper sensitivity. For instance, high Mn concentrations at the surface (Sunda 1989, Moffett 1997) could have decreased copper toxicity by lowering the Cu to Mn ratio (Sunda and Huntsman 1998a, Sunda and Huntsman 1998b). In contrast, high irradiance could increase copper toxicity by photodegrading L_1 and increasing the free Cu^{2+} in shallow mixed

layers. Exposure to UV radiation can also increase oxidative stress and membrane permeability, allowing more copper to enter the cell (Rai et al. 1995).

CONCLUSIONS

Prochlorococcus isolates were inhibited at free cupric ion concentrations that are not uncommon in the Sargasso Sea and that did not inhibit its close relative *Synechococcus*. Although all *Prochlorococcus* isolates were more sensitive to copper than *Synechococcus*, members of the low chl B/A (high light adapted) ecotype were less sensitive than strains with high chl B/A ratios (low light adapted). In the field the *in situ* concentration of free Cu^{2+} had a strong effect on the copper sensitivity of *Prochlorococcus*, which varied with depth and with season. *Synechococcus* were relatively copper resistant across a range of environments. Net growth rates were substantially reduced when *Prochlorococcus* from environments where the *in situ* free Cu^{2+} was low (deep mixed layers and below the thermocline in stratified water) were exposed to copper. *Prochlorococcus* in shallow mixed layers where *in situ* Cu^{2+} was high were less sensitive to copper and may have been members of the copper resistant low chl B/A ecotype. These data are consistent with the hypothesis that ambient copper levels may influence the relative abundance of *Prochlorococcus* and *Synechococcus* in the Sargasso Sea.

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Chapter 4

Prochlorococcus cell division rates in the Sargasso Sea.

ABSTRACT

In the northern Sargasso Sea the integrated abundance of *Prochlorococcus* and its distribution in the water column varies markedly over the seasonal cycle. During summer stratification, *Prochlorococus* numbers are low in the mixed layer but the integrated abundance is high due to a large subsurface population maximum. In the winter when the water column is well mixed, the cells are uniformly distributed with depth and the integrated abundance is lower than during the summer. In the permanently stratified southern Sargasso cell numbers are greater in the winter and the integrated abundance is higher than in the north. In order to gain some insight into the underlying mechanisms behind these changes in *Prochlorococcus* abundance *in situ* cell division rates were determined by analysis of DNA histograms. The impact of deep mixing was investigated by comparing cell division rates in winter and summer. In the well mixed water column, both abundance and cell division rates (0.1 to 0.2 day^{-1}) were low. Nutrient concentrations were relatively high, but cell division appeared to be limited by irradiance and temperature during the day examined. In both the seasonally stratified northern and permanently stratified southern Sargasso, cell division rates were systematically higher. Cell division rates in the southern Sargasso mixed layer were lower than those in the subtropical Pacific, probably because of more severe nutrient limitation.

INTRODUCTION

Prochlorococcus and *Synechococcus* are closely related marine cyanobacteria that account for a large fraction of phytoplankton biomass and productivity in subtropical and tropical seas. *Prochlorococcus* alone can account for 30% of the total chlorophyll in the Sargasso Sea, 25% of the yearly primary productivity (Goericke 1993) and 10 to 30% of the total living biomass (Buck K. R. 1996). These cyanobacteria respond to seasonal forcing differently and often have different temporal and spatial distributions. In environments where there is a strong pattern of seasonal forcing, the integrated abundance of *Prochlorococcus* declines in winter when the water column is well mixed and increases after summertime stratification. *Synechococcus* integrated abundance follows the opposite pattern. This has been well documented in the northern Sargasso Sea (Olson et al. 1990) and similar patterns have been seen in the Gulf of Aqaba (Lindell 1995), the Arabian Sea (Liu 1998) and the western tropical Pacific Ocean (Blanchot and Rodier 1996).

Vertical distributions of *Prochlorococcus* and *Synechococcus* also vary over the seasonal cycle in the northern Sargasso Sea. During the winter vertical profiles of these two groups of cyanobacteria are similar, with both groups evenly distributed in the well mixed water column. In the summer when the water column is stratified, *Prochlorococcus* forms a strong subsurface maximum below the mixed layer and few cells are left in the surface waters. *Synechococcus* are more uniformly distributed with depth throughout the year. As a result, even though the integrated abundance of

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Synechococcus is lower than that of *Prochlorococcus*, the former often outnumbers the later in shallow mixed layers (Olson et al. 1990, Andersen et al. 1996).

In the subtropical Pacific cyanobacteria distributions are significantly different. The water column is permanently stratified and *Prochlorococcus* are two orders of magnitude more abundant than *Synechococcus* in the surface waters throughout the year (Campbell and Vaulot 1993). There are several possible explanations for the differences in cyanobacteria distribution between the Atlantic and Pacific. Photoinhibition in shallow, well illuminated mixed layers is probably not the sole explanation. The stations sampled in the subtropical Pacific are 10 degrees further south (Liu et al. 1997) than those in the northern Sargasso Sea and therefore must be exposed to equal if not more irradiation since the mixed layer depths are comparable in the two environments (Kirk 1994, Liu et al. 1997). Nutrient depletion may also play a role because concentrations of soluble reactive phosphorous (SRP) are lower in the Atlantic than in the subtropical Pacific (Karl and Tien 1997, Cavender-Bares et al. submitted). Again this is probably not the entire story since the abundance of *Prochlorococcus* at the surface is 20 fold less in the seasonally stratified northern Sargasso in comparison to permanently stratified water columns further south (Chapter 3, see below), even though both inorganic nitrogen and SRP concentrations are very similar (Cavender-Bares et al. submitted). Finally, differential mortality could also influence picoplankton distributions (Olson et al. 1990, Appendix C).

Copper toxicity could also influence the distribution of cyanobacteria. For instance, there are significant differences in trace metal concentrations as well as cyanobacteria abundance in the northern subtropical Pacific and the Sargasso Sea. In the shallow mixed layers of the Sargasso Sea *Prochlorococcus* abundance is low and free Cu^{2+} can reach 6 pM, which is high enough to reduce the cell division rates of *Prochlorococcus* isolates in culture (Moffett et al. 1990, Moffett 1995, Chapter 3). In contrast, *Prochlorococcus* are abundant at the surface in the northern Pacific (Campbell and Nolla 1994, Liu et al. 1997) where the free Cu^{2+} is less than 0.2 pM (Coale and Bruland 1988, Coale and Bruland 1990).

To gain some insight into the underlying mechanisms behind differences in *Prochlorococcus* abundance in the Sargasso Sea, we measured *in situ* cell division rates in well-mixed, seasonally and permanently stratified water columns. The impact of deep mixing on cell division was determined by comparing cell division rates in the winter to those in stratified water columns in the summer. Cell division rates in stratified water columns in the Sargasso Sea were also compared to those in similar environments in the Pacific. *Prochlorococcus* cell division rates were measured by analysis of DNA histograms (McDuff and Chisholm 1982, Carpenter and Chang 1988, Vaulot et al. 1995). This method is based on *in situ* sampling and thus avoids the problem of bottle effects.

METHODS

Field samples for cell division rate measurements.

In situ samples were collected at various depths from three stations in the Sargasso Sea using Niskin bottles mounted on a CTD rosette. The locations and descriptions of each water column are summarized in Table 1 and Fig 1. The stations were chosen to represent a range of environments with different levels of physical forcing and included well mixed, seasonally stratified and permanently stratified water columns. Samples were taken every one to three hours over a 24 hour light-dark cycle. In order to ensure that the same water mass was being sampled over this time period, cast locations were determined by following a buoy attached to a drouge with 25m of line. All samples were preserved in a final concentration of 0.1% glutaraldehyde for 10 minutes at room temperature in the dark and were then stored in liquid nitrogen (Vaulot et al. 1989).

Table 1) Summary of cruise locations and water column characteristics.

Cruise	Date	Location	Description
OC297	January, 1997	Northern Sargasso 35° 42 N, 62° 57 W	Well mixed 200m mixed layer
OC325	June, 1998	Northern Sargasso 31° 40 N, 64° 07 W	Seasonally stratified 25m mixed layer
OC318	February, 1998	Southern Sargasso 26° 00 N, 70° 00 W	Permanently stratified 75m mixed layer
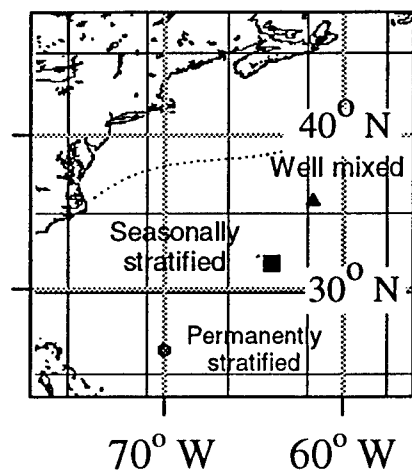
Fig 1) Map of stations sampled in the Sargasso Sea. Different symbols represent different cruises. The January 1997 station was well mixed, the June 1998 station was seasonally stratified and the February 1998 station was permanently stratified.

DNA staining and flow cytometry.

Potassium citrate (final concentration 30 µM) and a $1:10^{-4}$ final dilution of the DNA specific stain Sybr Green I (Molecular Probes) were added to thawed samples and incubated at room temperature in the dark for 15 minutes before analyzing by flow cytometry. All working stocks of Sybr Green I were diluted in DMSO (Marie et al. 1997). In the seasonally stratified water column, *Prochlorococcus* chlorophyll fluorescence in the upper 30m was dim. As a result, it was not possible to separate these cells from heterotrophic bacteria when the samples were stained with Sybr Green, so no DNA histograms from 15 or 30m are available.

The amount of DNA per cell, as well as *Prochlorococcus* number and forward angle light scatter (FALS, which is a measure of cell size), were quantified using a modified EPICS 753 flow cytometer as described in Binder et al (1996) except that the instrument was used in single beam mode and the optical filters were arranged to detect green fluorescence from Sybr Green I, which is excited at 488 nm. After passing through 630 and 560 nm short-pass dichroic filters, green signals passed though a 515 nm long-pass filter. Samples were delivered at a constant rate using a syringe pump (Harvard Apparatus, Model 22). The lowest coefficients of variation (CV) in terms of fluorescence were achieved by using low sample flow rates (5 to 7 µl min^{-1}) and sample lines which were pre-equilibrated with the stain for at least one hour.

For a summary of the terms used in calculating cell division rates see the glossary of important terms (see below). The cell cycle of *Prochlorococcus* is similar to the eukaryotic cell cycle in that it has a discrete DNA synthesis phase, even when the cells are growing faster than one division per day (Shalapyonok et al. 1998). As a result, the eukaryotic terms G_1, S, and G_2 + M are often applied to the *Prochlorococcus* cell cycle for the sake of convenience. Moreover, cell division in *Prochlorococcus* is tightly synchronized to the light dark cycle (Vaulot et al. 1995, Liu et al. 1997, Shalapyonok et al. 1998) which facilitates cell cycle analysis. In order to determine the fraction of cells

Glossary of important terms

Parameter	Units	Definition
μ	day^{-1}	Cell division rate: the growth rate in the absence of cell losses.
μ_{dna}	day^{-1}	Calculated from cell cycle analysis using Eq. 1.
μ_{min}	day^{-1}	The minimum cell division rate, calculated from DNA histograms taken at one time point using Eq. 2.
$f(S+G_2)_t$	unitless	Fraction of cells in the S and G_2+M phases of the cell cycle at time t.
$t_{(S+G2)}$	days	Duration of the S and G_2+M phases.

in each cell cycle phase, DNA fluorescence histograms were modeled and divided into

G_1, S, and G_2+M using ModFit software (Verity Software House, Inc.).

Following the method of Liu et al. (1997), cell division rates (μ_{dna} day^{-1}) were obtained

from an analysis of DNA histograms over the entire light dark cycle using

$$\mu = \frac{1}{t_{(S+G_2)}} \int_{0 \text{ hrs}}^{24 \text{hrs}} \ln[1 + f(S + G_2)_t] \qquad (1)$$

where $f(S+G_2)_t$ is the fraction of cells in the S and G_2+M phases of the cell cycle at time

t, and $t_{(S+G_2)}$ is the time (in hours) required for cells to pass through S and G_2 + M. The

latter can be estimated as twice the distance between the peak of cells in the S phase and

the peak of cells in the G_2+M phase (McDuff and Chisholm 1982, Carpenter and Chang

1988, Vaulot et al. 1995, Liu et al. 1997). Using this approach the $t_{(S+G_2)}$ values for the

permanently stratified water column were 7.0 and 3.5 hours at 50 and 125m, respectively.

Progression through the cell cycle required more time in the well mixed and seasonally

stratified stations; the $t_{(S+G_2)}$ values were 9.3 and 9.5 hours, respectively (Table 2).

These agree well with the 8 to 9 hours required in the northeastern tropical Atlantic

(Partnesky et al. 1996) (The $t_{(S+G_2)}$ used by Partensky (1996) is 4.5 hours, but

examination of the data supports the longer values mentioned here). Shorter cell cycle

duration times comparable to those found in the permanently stratified water column

have been measured in the subtropical Pacific (6 hrs, Liu et al. 1997), the equatorial

Pacific (4.3 to 6 hrs, Vaulot et al. 1995, Mann and Chisholm in press, Chapter 3) and the Arabian Sea (2 to 3 hrs, Shalapyonok et al. 1998).

Since the $t_{(S+G_2)}$ values have a strong impact on the cell division rate estimate, a more conservative analysis of the minimum possible cell division rate was calculated. Since the *Prochlorococcus* cell cycle is synchronized and all cells are in G_1 in the morning, any cells present in S or G_2+M in the evening must divide sometime during the night. A minimum cell division rate can then be obtained by determining the fraction of cells in S or G_2+M and using equation 2 (Binder et al. 1996).

$$\mu_{min} = \ln[1 + f(S + G_2)_t] \qquad (2)$$

At least this number of cells must divide, but in order to avoid seriously underestimating the cell division rate it is important to determine the peak of cells in S or G_2+M (Binder et al. 1996). Because the timing of cell division varies with depth, DNA histograms from several time points were analyzed and the timepoint that gave the highest μ_{min} estimate was used.

RESULTS AND DISCUSSION

Depth profiles of temperature and in situ fluorescence.

The seasonal cycle has a strong impact on the physical characteristics of the water column in the northern Sargasso Sea (Olson et al. 1990, Siegel 1990, Goericke 1993, Bidigare 1990, Deuser 1986). In our study the mixed layer in the winter was approximately 200m; temperature was generally uniform throughout this depth at 18 to 19°C, but there was a very slight break in temperature at 100m (Fig 2A). A well developed subsurface maximum in chlorophyll fluorescence was not present (Fig 2A). In the summer, the water column was well stratified with a mixed layer of 25m and some partial stratification at 10 to 12m. A strong subsurface maximum in chlorophyll fluorescence was located below the thermocline. (Fig 2B). The physical characteristics of the permanently stratified water column in the southern Sargasso were similar, except the mixed layer was deeper at 75m. The *in situ* chlorophyll fluorescence maximum occurred within the thermocline about 20m deeper than in the seasonally stratified water column (Fig 2C).

Cyanobacterial abundance.

Picoplankton distribution in the Atlantic has been well studied and our data is in agreement with earlier results (Olson et al. 1990, Goericke 1993, Veldhuis. M. J. 1993, Partensky et al. 1996). In the northern Sargasso when the mixed layer was deep (200m),
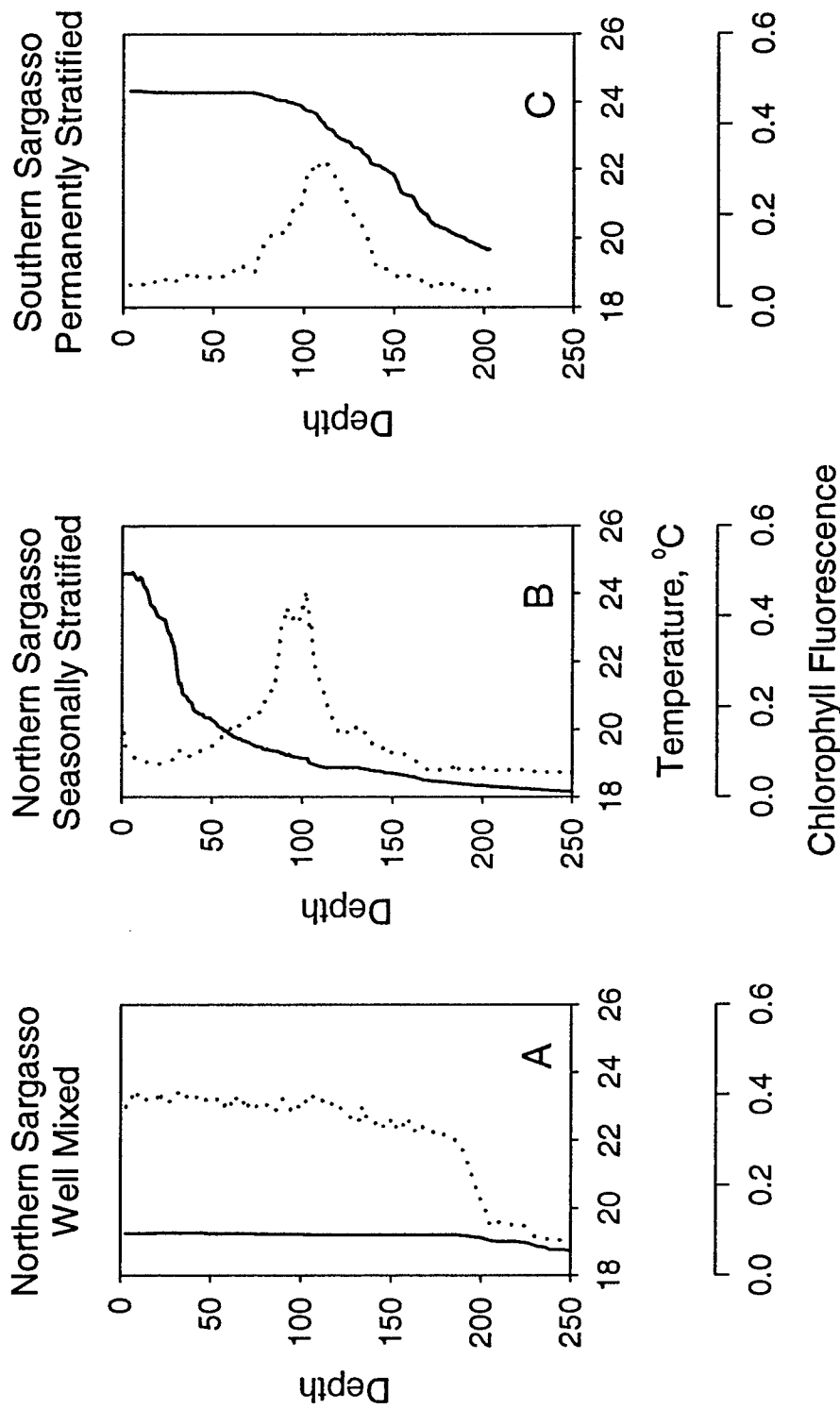
Fig 2) Depth profiles of temperature (solid line) and relative chlorophyll fluorescence (dotted line) for **A)** the well mixed and **B)** seasonally stratified water columns in the northern Sargasso Sea. **C)** Data from the permanently stratified water column in the southern Sargasso. Note that the chlorophyll fluorescence scales are relative and not directly comparable in A, B, and C.
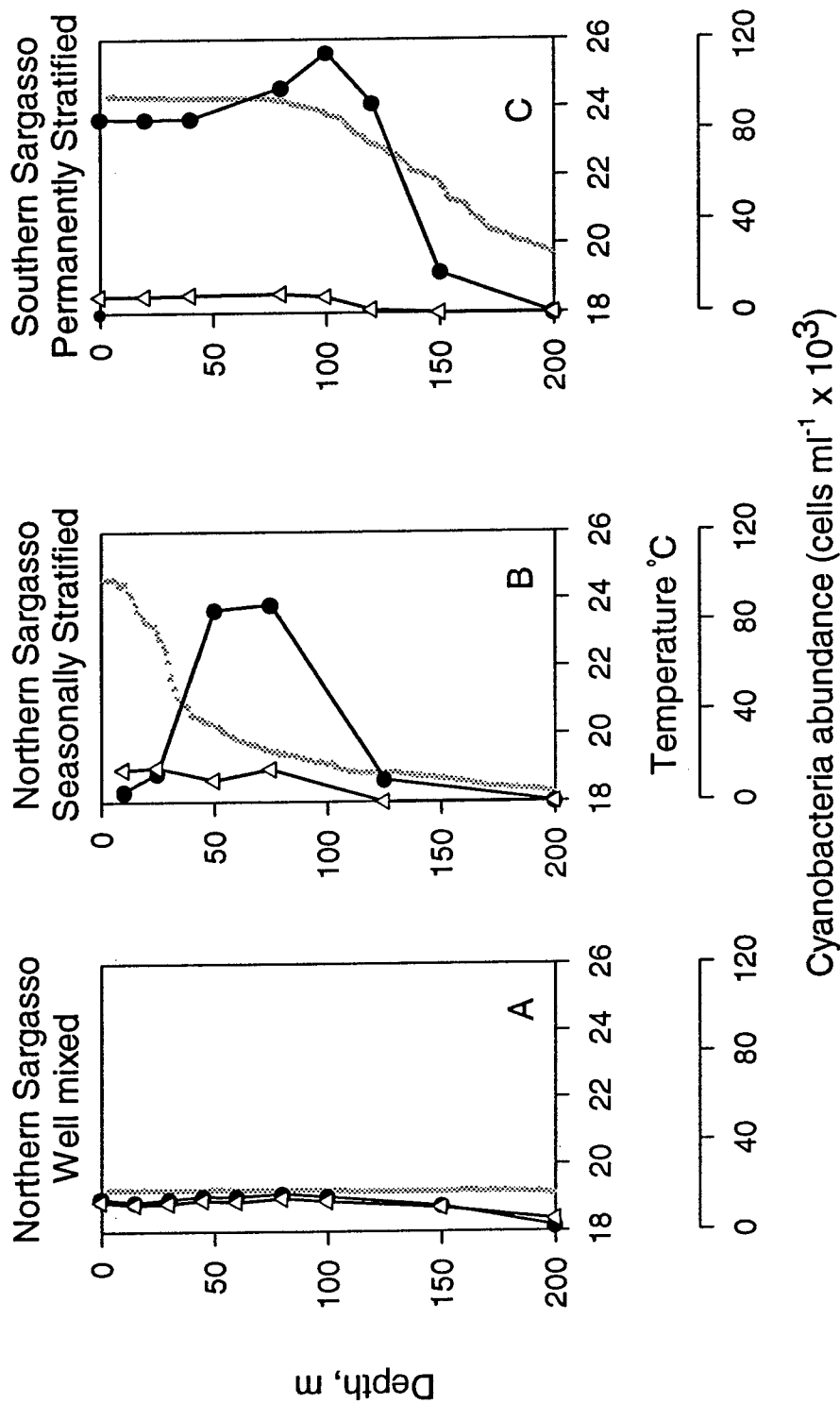
Fig 3) Depth profiles of *Prochlorococcus* (black circles) and *Synechococcus* (white triangles) abundance with temperature (grey line) from **A)** the well mixed and **B)** seasonally stratified northern Sargasso, and C) the permanently stratified southern Sargasso.

the concentrations of *Prochlorococcus* and *Synechococcus* were roughly equal (1.5×10^4

ml^{-1}) and constant until cell numbers began to decline between 100 and 200m (Fig 3). As

stratification increased and the mixed layer became progressively shallower,

Prochlorococcus abundance decreased at the surface but a well-developed population

maximum developed below the mixed layer (Fig 3). In the southern Sargasso, the highest

Prochlorococcus numbers were found below the mixed layer. However, cell numbers

declined only slightly towards the surface and *Prochlorococcus* were significantly more

abundant than *Synechococcus* within the mixed layer (Fig 3).

In all three regimes, *Synechococcus* abundance was uniform with depth until below 75 to

100m when the population began to decline. This pattern was unchanged even when the

mixed layer depth varied from 25 to 200m in the northern Sargasso. The only difference

was the greater penetration of *Synechococcus* at depth in the well mixed water column

(Fig 3). The integrated abundance of *Synechococcus* followed the opposite pattern as that

of *Prochlorococcus*. Instead of being the most abundant in the permanently stratified

station, it was greatest in the northern Sargasso, especially in the winter when

stratification broke down (Fig 3).

Cell cycle progression patterns

The typical *Prochlorococcus* cell cycle pattern was followed in the permanently stratified

water column (Vaulot et al. 1995, Partnesky et al. 1996, Liu et al. 1997, Liu 1998, Mann

and Chisholm in press, Chapter 2). During the day at both 50 and 125m, cells were in G_1

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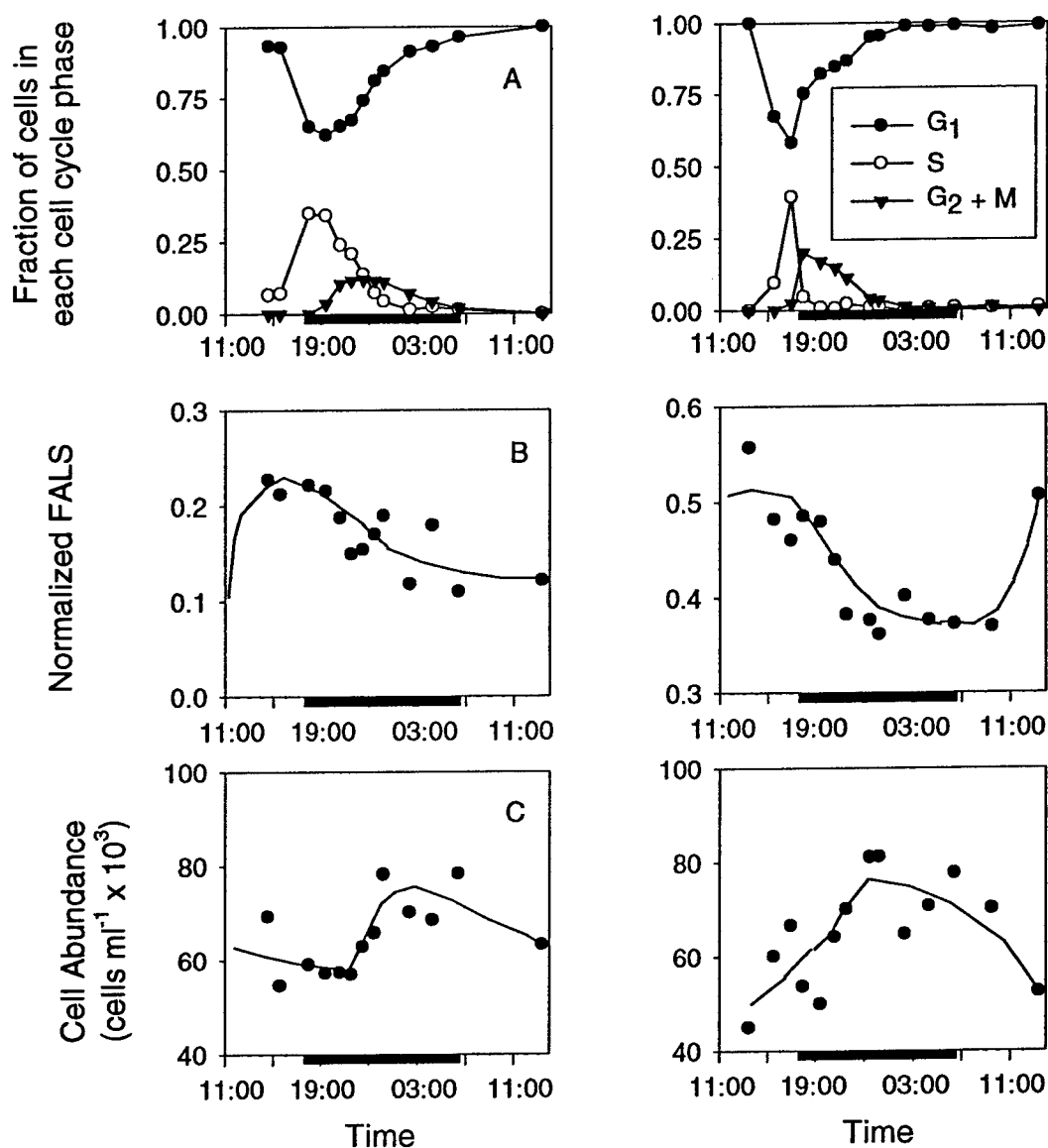
Fig 4) Diel patterns seen in *Prochlorococcus* for samples from 50m (left panel) and 125m (right panel) in a permanently stratified water column in the southern Sargasso Sea. The mixed layer depth was 75m. **A)** Fraction of cells in each cell cycle phase, **B)** FALS per cell normalized to standard beads and **C)** cell abundance over the light dark cycle. The solid bar represents the dark period.

and cell numbers declined as a result of grazing and other losses (Fig 4A). DNA synthesis started in the late afternoon and was followed by a peak of cells in G_2+M. As has been observed by others (Vaulot et al. 1995, Liu et al. 1997, Vaulot 1999), DNA synthesis was delayed in the mixed layer, perhaps avoiding UV damage to DNA (Vaulot et al. 1995). For instance, the maximum fraction of cells in the S phase was similar throughout the upper 125m at 34 to 39%, but occurred about one hour later at 50m compared to below the mixed layer (Fig 4A). Within the mixed layer the time required to progress through the cell cycle ($t_{(S+G_2)}$) was twice the 3.5 hours required at 125m (Table 2). It is unusual for $t_{(S+G_2)}$ to decrease with depth, in most cases cell cycle progression gets longer as light becomes less available (Vaulot et al. 1995, Liu et al. 1997). It may be possible that cell division was synchronized less tightly within the 75m mixed layer, this may broaden the cell cycle phase curves and lead to an overestimation of $t_{(S+G_2)}$.

As cell division takes place, mean FALS per cell should decrease (Fig 4B) and cell numbers should increase (Fig 4C). This pattern is loosely evident at 125m. FALS per cell was more variable at 50m, but did decline from the peak of cells in S in the afternoon until cell division was complete in keeping with the predicted pattern (Fig 4C). The amplitude of the FALS change over the diel cycle ($FALS_{max}/FALS_{min}$) was 1.4 in the deeper water and 1.6 at 50m (Table 2). If cell division is synchronized to the same extent, cells with the greatest change in FALS have the highest cell division rate (DuRand 1995, Binder et al. 1996). Thus, the $FALS_{max}/FALS_{min}$ ratio is a qualitative measure of the cell division rate.
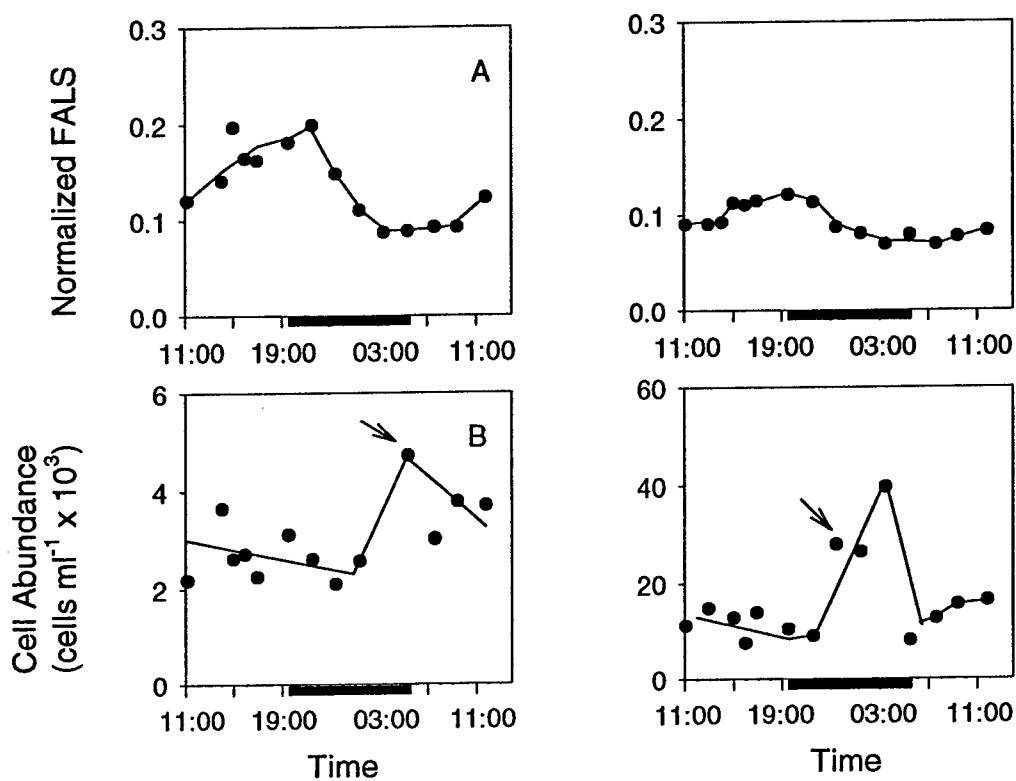
Fig 5) Diel patterns seen in *Prochlorococcus* for samples from 15m (left panel) and 30m (right panel) in a seasonally stratified water column in the northern Sargasso Sea. The mixed layer depth was 25m. **A)** FALS per cell normalized to standard beads and **B)** cell abundance over the light dark cycle. The solid bar represents the dark period. Arrows in **B)** point out large increases in cell number that may be due to the shoaling of the thermocline (see text).

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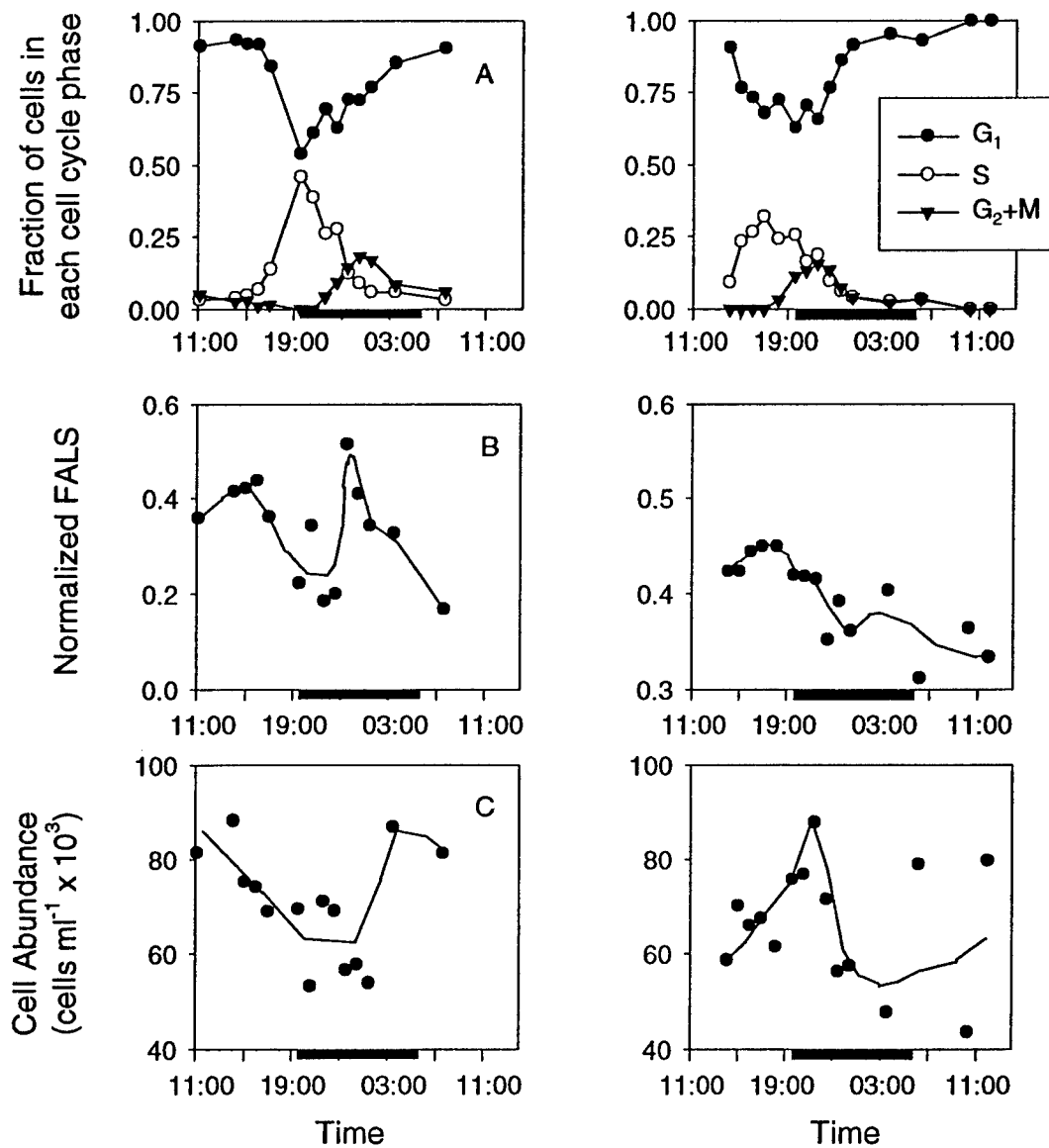
Fig 6) Diel patterns seen in *Prochlorococcus* for samples from 80m (left panel) and 100m (right panel) in a seasonally stratified water column in the northern Sargasso Sea. The mixed layer depth was 25m. **A)** Fraction of cells in each cell cycle phase, **B)** FALS per cell normalized to standard beads and **C)** cell abundance over the light dark cycle. The solid bar represents the dark period.

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In the seasonally stratified water column we were unable to measure DNA per cell in the samples from 15 and 30m from because the red autofluorescence of *Prochlorococcus* was too dim to distinguish them from heterotrophic bacteria. However, it was apparent from the diel patterns in cell density and FALS per cell that cell division was synchronized (Fig 5A and B). Deeper in the water column at 80 and 100m pigment fluorescence per cell increased and we were able to measure the DNA content per cell. In some respects the results were similar to those in the permanently stratified water column (Figs 4 and 6). For instance, the maximum fraction of cells in S phase was 46 and 32% at 80 and 100m (Fig 6A), compared 34 to 39% in the upper 125m in the permanently stratified station. However, the time required to progress through the cell cycle (9.5 hours) in the seasonally stratified water column was significantly longer than the $t_{(S+G_2)}$ values in the permanently stratified water column (Table 2). Cell cycle parameters in the seasonally stratified water column were also probably affected by the passage of an internal wave (see below) and should be viewed as a rough average over at least 10 meters.

Patterns of abundance and FALS per cell in the seasonally stratified water column were highly variable (Figs 5 and 6). For instance, cell numbers rapidly increased by a factor of 3 between 23:30 and 3:30 at 30m (Fig 5B). To account for this increase in abundance, even if cell losses were zero, these *Prochlorococcus* would have to be dividing more than twice a day. This is unlikely, since cell division rates greater than 0.69 day^{-1} (one division per day) have been found only in the highly productive Arabian Sea (Shalapyonok et al. 1998), or in iron enriched areas of the equatorial Pacific (Mann and Chisholm in press, Chapter 2).

A more probable explanation for this pattern is the shoaling of the thermocline that occurred between 23:30 and 3:30. Depth profiles of temperature and chlorophyll fluorescence indicate that the thermocline and fluorescence maximum shifted up by approximately 10m (Fig 7) during this time interval, probably because of an internal wave (for similar data see Partensky et al. 1996). Because of the sharp gradients in *Prochlorococcus* cell number, effectively sampling even a few meters lower in the water column would have simultaneously increased cell numbers at the surface and decreased them with depth. Some of these fluctuations in cell number and FALS could also have been caused by sampling different parcels of water. A drouge was followed during the experiments to minimize this, but it was set at 25m so at the deeper depths water with different histories may have been sampled.

Patterns in mean FALS over the light dark cycle could also be influenced by the shoaling of the thermocline, since FALS increased four fold between 15 and 80m, and should therefore be treated with caution. However, there were some very rough trends (Fig 6B and C). The $FALS_{max}/FALS_{min}$ ratio ranged between 1.7 and 3.0 in the upper 80m and then dropped to 1.4 at 100m (Table 2). DuRand (1995) also found that $FALS_{max}/FALS_{min}$ ratios were lower at deeper depths, suggesting lower cell division rates.

In the well mixed water column, changes in cell cycle parameters throughout the light dark cycle were muted compared to those in the stratified water columns (Fig 8),
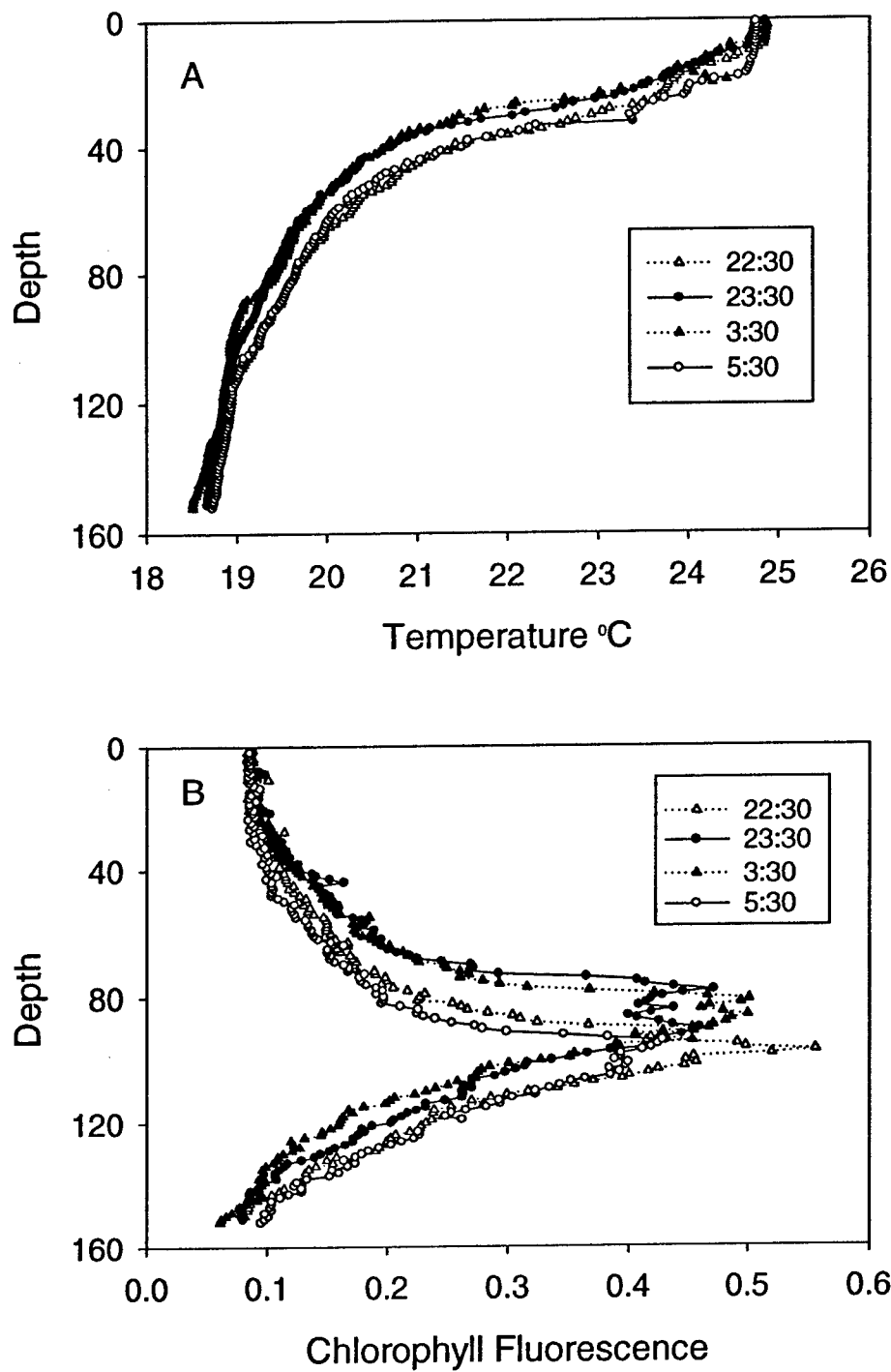
Fig 7) Depth profiles of **A)** temperature and **B)** relative chlorophyll
fluorescence from 22:30 to 5:30 in the seasonally stratified water column.
Note the shoaling of both the thermocline and chlorophyll fluorescence
maximum between 23:30 and 3:30.
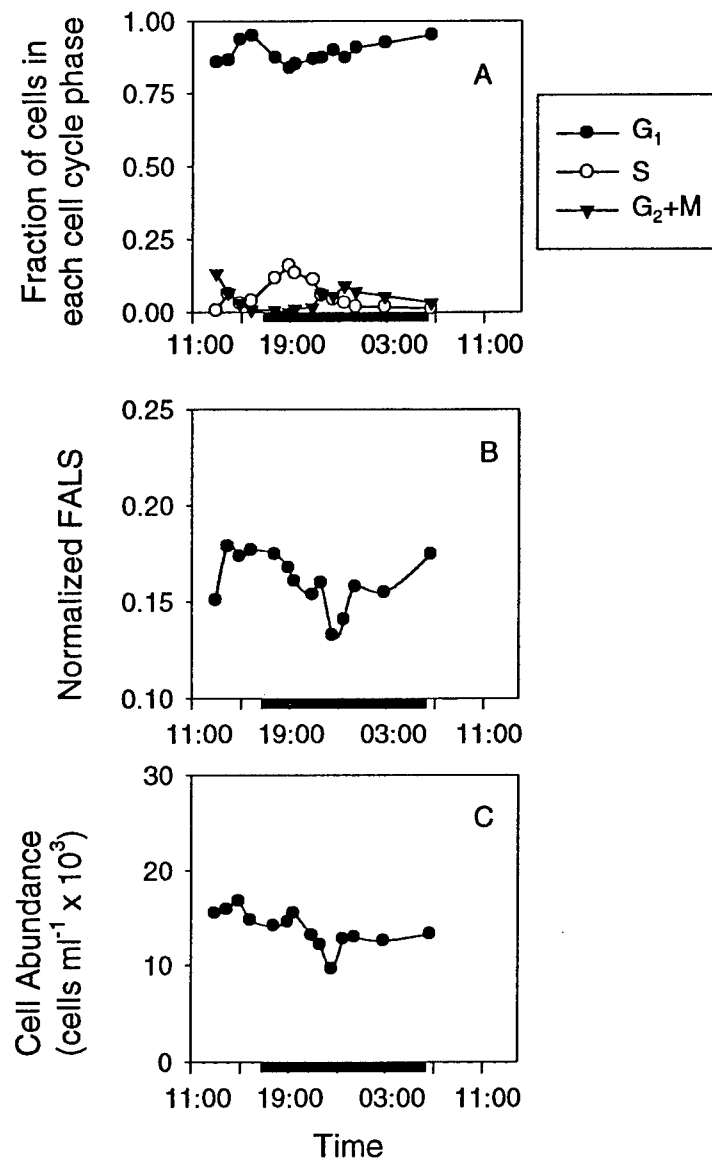
Fig 8) Diel patterns seen in *Prochlorococcus* for samples from 45m in a well mixed water column in the northern Sargasso Sea. The mixed layer depth was 200m.
A) Fraction of cells in each cell cycle phase, **B)** FALS per cell normalized to standard beads and **C)** cell abundance over the light dark cycle. The solid bar represents the dark period.

suggesting lower cell division rates. At 45m the maximum number of cells in the S phase was 16% of the total population(Fig 8A), which is at least half the values seen in the stratified stations. $FALS_{max}/FALS_{min}$ ratio of 1.3 at 45m was similar to those found at 100 or 125m in the stratified water columns(Fig 8B, Table 2). Cell numbers decreased during the day as expected, but there was generally no increase in cell number later at night when the DNA histograms indicated division was occurring (Fig 8A and C).

An absence of large variations in cell cycle parameters over the diel cycle could result from both low cell division rates and a lack of synchrony in cell division. Changes in cell cycle parameters were not large, but they were sufficient to indicate that cell division was synchronized to some extent. There were clearly recognizable peaks of cells in S and G_2+M, the fraction of cells in S phase ranges from 0 to 16%. If cell division had been completely asynchronous the fraction of cells in any cell cycle phase would remain constant during the light dark cycle. The $t_{(S+G_2)}$ of 9.3 hours was also similar to the time required to progress through the cell cycle in the seasonally stratified water column, where cell division was clearly synchronized.

Cell division rates.

One current view of cyanobacteria ecology maintains that cell division rates are regulated by inter-relationships between irradiance, nutrients, temperature and possibility copper toxicity (Chapter 3). To gain insight into how the relative importance of each of these factors changes with season and with depth cell division rates were calculated by two

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methods. Minimum cell division rates were calculated from the fraction of cells

committed to division at a particular time (Eq. 2, μ_{min}). An analysis of DNA histograms

throughout the cell cycle was used to determine the cell division rate using Eq. 1 (μ_{dna}).

μ_{min} and μ_{dna} agreed within 10% (on average) and the largest difference between the two

was 25% (Table 2). Liu et al found similar differences between μ_{min} and μ_{dna} (Liu 1999).

Cell division rates below the mixed layers in the stratified water columns were

comparable to those in the subtropical Pacific at similar depths (Liu et al. 1997). In the

permanently stratified water column μ_{min} was 0.4 day^{-1} at both 80 and 125m while μ_{dna} at

125m was also relatively high at 0.5 day^{-1} (Table 2, Fig 9). In the seasonally stratified

Table 2) Summary of cell cycle parameters and cell division rates, day^{-1}.

Station	Depth	$FALS_{max}/$ $FALS_{min}$	$t_{(S+G2)}$	μ_{min}	μ_{dna}
Northern	0			0.2	
Sargasso:	15			0.1	
well mixed	45	1.3	9.3	0.2	0.1
	80			0.1	
	150			0.0	
Northern	15	2.3			
Sargasso:	30	1.7			
seasonally	80	3.0	9.5	0.4	0.4
stratified	100	1.4	9.5	0.3	0.3
Southern	0			0.3	
Sargasso:	50	1.6	7.0	0.3	0.4
permanently	80			0.4	
stratified	125	1.4	3.5	0.4	0.5
	150			0.2	

water column, μ_{dna} at 80m was similar at 0.4 day^{-1} (Table 2, Fig 10). Relatively high cell

division rates at depth may be explained by the presence of *Prochlorococcus*

subpopulations adapted to low light environments (Campbell and Vaulot 1993, Partensky et al. 1993, Partnesky et al. 1996, Moore 1997, Moore et al. 1998). This conclusion is supported by the fact that cells below the mixed layer were more copper sensitive than those at the surface. This is important because cultured isolates that are sensitive to copper are also members of the low light adapted, high chl B/A ecotype (Chapter 3). In both stratified water columns there is a trend of decreasing cell division rates at depths where *Prochlorococcus* abundance was also decreasing. This occurred at 125m in the permanently stratified water column and at 100m in the seasonally stratified water column (Table 2, Figs 9 and 10).

Within the mixed layer of the permanently stratified southern Sargasso Sea, minimum cell division rates were 0.3 day^{-1} (Fig 9, Table 2) and were similar to the μ_{dna} at 50m of 0.4 day^{-1}. These cell division rates are significantly lower than the rates of 0.6 day^{-1} or higher in the subtropical Pacific at similar depths (Liu et al. 1997). One explanation for the lower cell division rates in the southern Sargasso is that nutrient concentrations, especially of phosphorous, are lower in the Sargasso Sea than in the Pacific (Karl and Tien 1997, Cavender-Bares et al. submitted).

It is unlikely that cell division rates in the southern Sargasso were significantly light limited. In the southern Sargasso the mixed layer (75m) was two to three times deeper and samples were taken earlier in the year (February as opposed to September and July) compared to the subtropical Pacific (Liu et al. 1997). However, the difference in average daily insolation between September in the subtropical Pacific and February in the
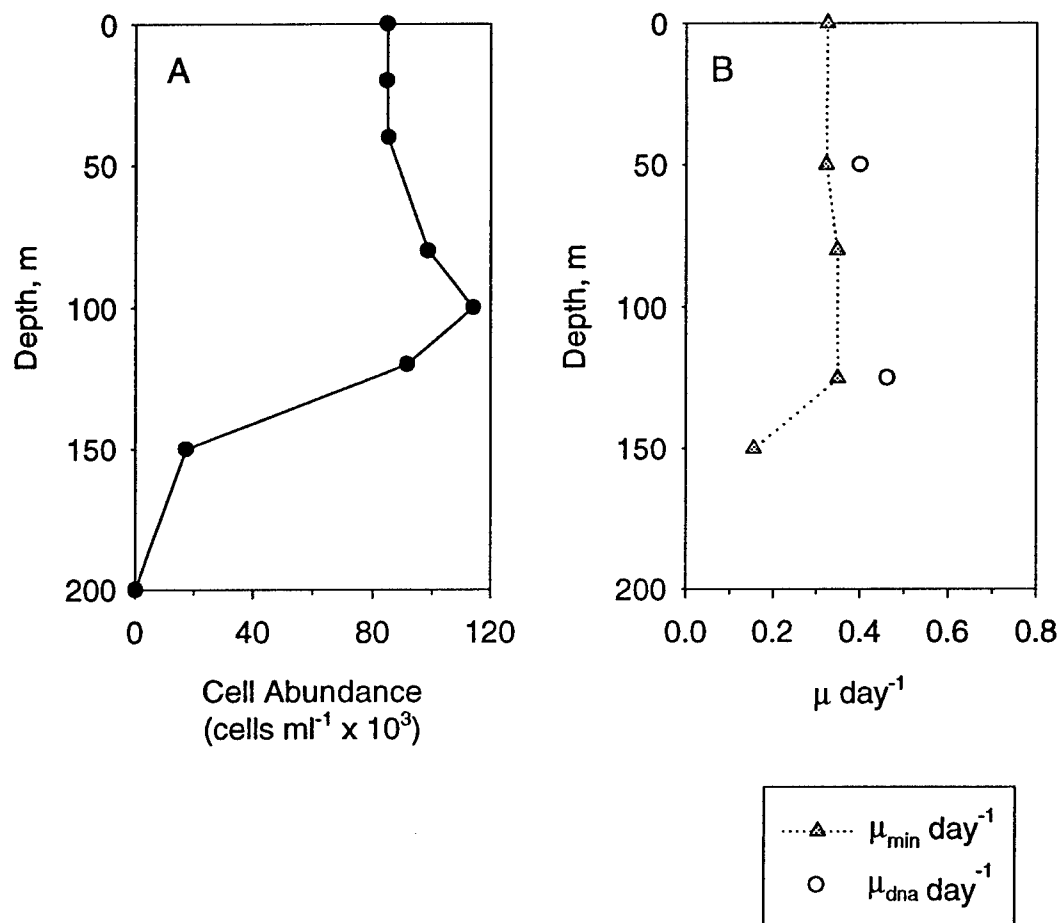
Fig 9) Depth profiles of **A)** *Prochlorococcus* abundance and **B)** cell division rates in a permanently stratified water column in the southern Sargasso Sea. μ_{min} and μ_{dna} day^{-1} are both based on an analysis of DNA histograms. μ_{min} is a robust estimate of the minimum possible cell division rate from one time point, while μ_{dna} is determined by analyzing DNA histograms over the entire light dark cycle.
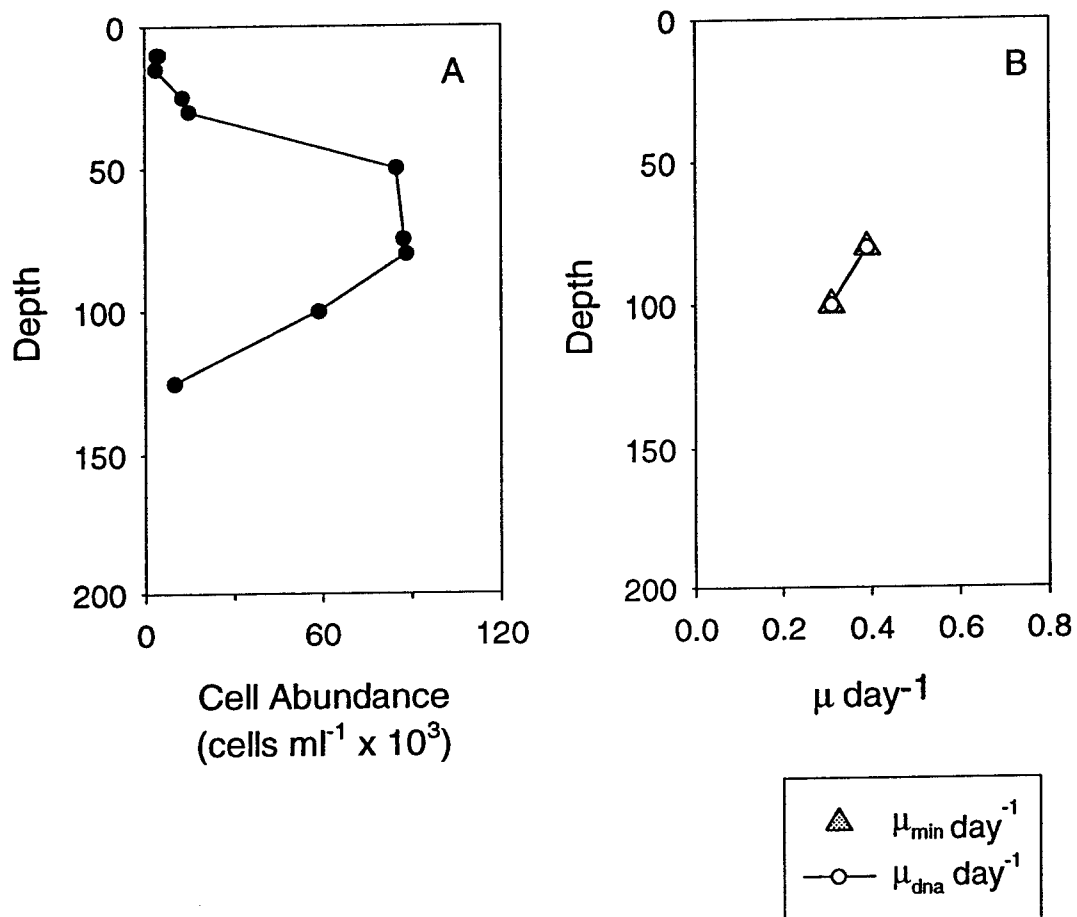
Fig 10) Depth profiles of **A)** *Prochlorococcus* abundance and **B)** cell division rates in a seasonally stratified water column in the northern Sargasso Sea. μ_{min} and μ_{dna} day^{-1} are both based on an analysis of DNA histograms. μ_{min} is a robust estimate of the minimum possible cell division rate from one time point, while μ_{dna} is determined by analyzing DNA histograms over the entire light dark cycle.

southern Sargasso is less than 10% (Kirk 1994). Maximum cell division rates in the

subtropical and equatorial Pacific also occur between 40 to 70m (Vaulot et al. 1995, Liu

et al. 1997, Vaulot et al. 1999), not at the surface. Finally, cell division rates below the

mixed layer in the southern Sargasso were equal to or higher than those in the subtropical

Pacific. If differences in irradiation were large between these two environments, one

would expect that the cell division rates deep in the water column would also be different.

Prochlorococcus are also sensitive to copper toxicity (Chapter 3), which could also

reduce cell division rates in the Sargasso Sea in stratified water columns. Free cupric ion

concentrations (free Cu^{2+}) measured in control bottles from within the mixed layers of the

permanently and seasonally stratified water columns were 2 and 3.6 pM, respectively

(Chapter 3). These values are significantly higher than free Cu^{2+} measured in the Pacific

which are below 0.2 pM (Coale and Bruland 1990).

However, the effect of copper toxicity on cell division rates measured in this study is not

clear – even by inference. In the permanently stratified water column, other explanations

such as nutrient limitation could explain the relatively low cell division rates in the mixed

layer. In the mixed layer of the stratified water column, where *Prochlorococcus* are 20x

less abundant and the free Cu^{2+} concentration is high (Chapter 3), cell division rate could

not be determined in this study using DNA analysis. The shoaling of the thermocline

could have also influenced cells numbers and FALS per cell – making estimates of cell

division rates using these parameters in this water column untenable. Cell division rates

may have decreased slightly at the surface. Reduced cell division rates in shallow mixed

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layers have been seen in the subtropical (Liu et al. 1997) and equatorial Pacific (Vaulot et al. 1995). In the Sargasso Sea, cell division rates estimated using the $FALS_{max}/FALS_{min}$ ratio were generally fairly uniform with depth, with a slight trend of decreasing cell division rates toward the surface (DuRand 1995). Cell division rates in the Sargasso Sea measured by ^{14}C incorporation into taxon-specific pigments were more variable. Decreases in cell division rates in the surface were observed, but in only half of the stratified water columns examined (Goericke 1993).

In the well mixed water column cell division rates were systematically lower than in the stratified stations. This difference could be due to the cloudy weather on the day of sampling as well as on the depth of the mixed layer. In the upper 80m, minimum cell division rates (μ_{min}) ranged from 0.1 to 0.2 day^{-1} (Fig 11). μ_{dna} at 45m was 0.1 day^{-1}, 4x lower than μ_{dna} values at similar depths in the stratified water column (Table 2, Fig 11). Constant cell division rates in the upper 80m were not surprising given the extent of the mixed layer and the uniform cell numbers with depth (Figs 2 and 11). These rates were generally comparable in magnitude with the rates measured in other studies. Goericke (1993) reported a mean cell division rate of approximately 0.25 day^{-1} for *Prochlorococcus* in the Sargasso Sea using ^{14}C incorporation into taxon-specific pigments, but found no systematic differences in *Prochlorococcus* cell division rates between well mixed and stratified water columns. This may be a reflection of variability in both the weather and the techniques used to measure cell division rates. It is possible that cell division rates would have been higher in the well mixed water column if more light was available. Cell division rates estimated using the ^{14}C incorporation method

Fig 11) Depth profiles of **A)** *Prochlorococcus* abundance and **B)** cell division rates in a well mixed water column in the northern Sargasso Sea.

μ_{min} and μ_{dna} day^{-1} are both based on an analysis of DNA histograms.

μ_{min} is a robust estimate of the minimum possible cell division rate from one time point, while μ_{dna} is determined by analyzing DNA histograms over the entire light dark cycle.
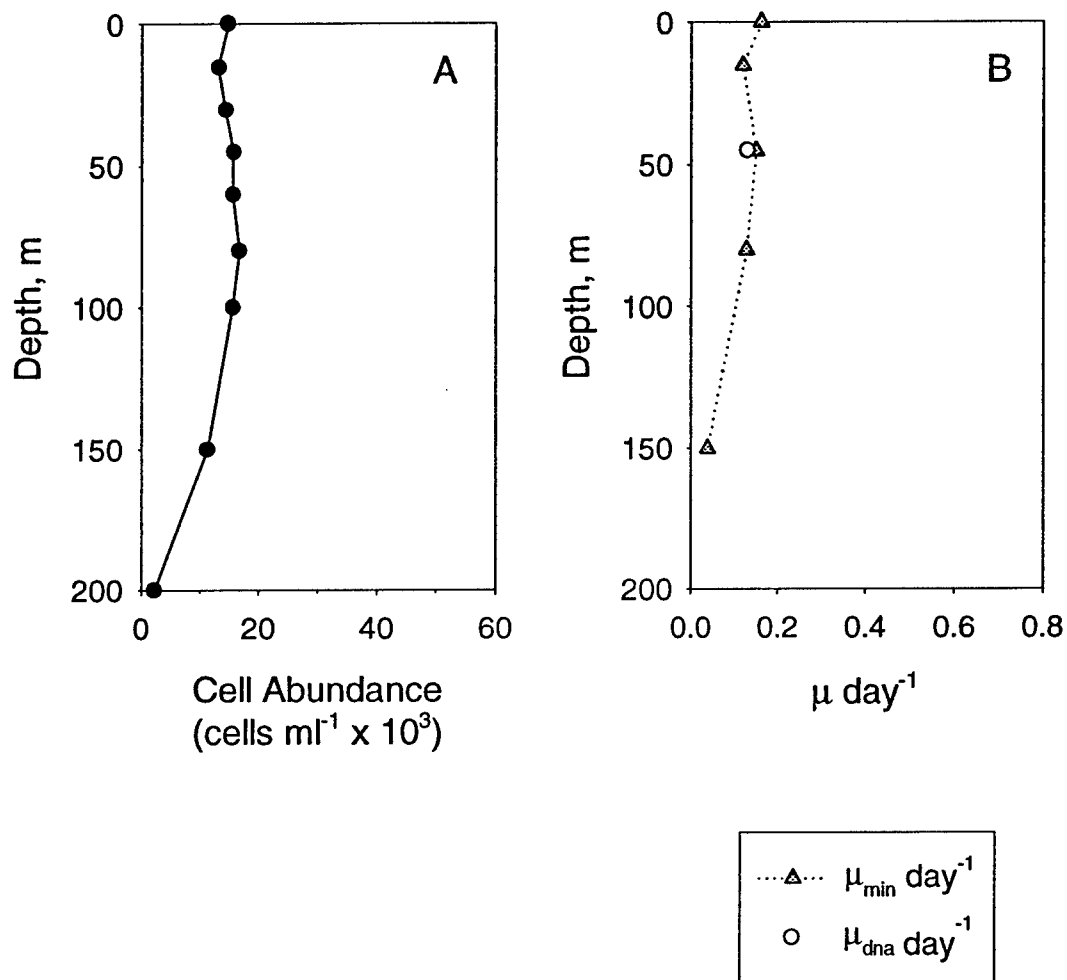
could also have been over or underestimated if the cyanobacteria were photoadapting during the bottle incubations with labeled carbon (Goericke 1993).

Cell division rates in the well mixed water column were probably limited by the available irradiance and temperature. When water from 45m was incubated on deck with stable, constant illumination the net growth rate of *Prochlorococcus* was 0.4 day^{-1} (Fig 12). Since the μ_{dna} *in situ* was only 0.1 day^{-1}, the cell division rate in the bottle must have increased (See Eq. 4). This high net growth rate indicates that nutrients were not limiting; nitrogen and phosphate concentrations were 100 and 3 fold higher , respectively, at the surface during the winter than they were in the stratified water columns (Cavender-Bares et al. submitted). The free Cu^{2+} concentration in the bottle of 0.6 pM also did not prevent the cell division rate from increasing (Chapter 3). The most likely explanation for the doubling (at least) of the cell division rate in the bottles is that the cells *in situ* were light limited. The day was overcast and average insolation in winter is about half that in summer at this latitude (Kirk 1994). The importance of light limitation is also supported by the fact that the chlorophyll fluorescence per cell decreased significantly during the on-deck incubations (data not shown), which implies that the light availability increased when the cells were removed from the deep mixed layer and placed in a stable environment. Temperature could also play a role in reducing cell division rates. Cell division rates of *Prochlorococcus* isolates in the laboratory are highest at a temperature of 24°C. In the 18°C well mixed water column, cell division rates could be reduced by about 40% (Moore et al. 1995).
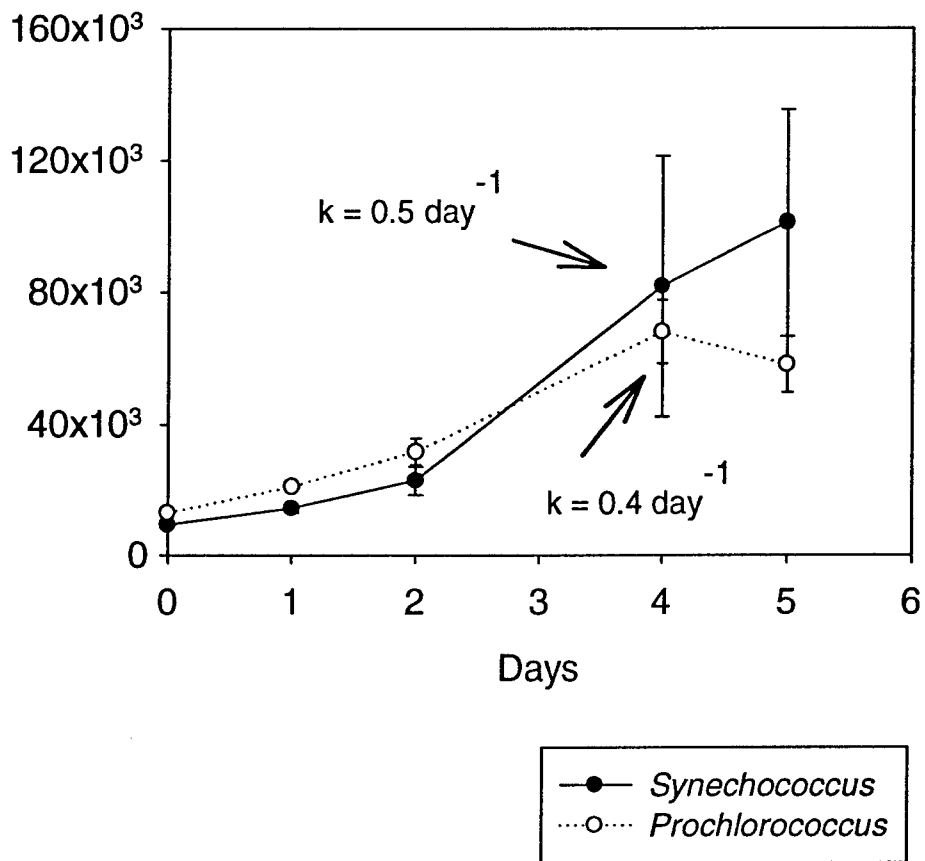
Fig 12) *Prochlorococcus* and *Synechococcus* abundance during an on-deck bottle incubation of water from 45m in the well mixed water column. The net growth rates calculated during the first four days are noted.

CONCLUSIONS

In the northern Sargasso Sea seasonal forcing is strong and deep mixed layers in the winter months alternate with highly stratified water columns in the summer. *Prochlorococcus* abundance changes markedly during this seasonal cycle. During the winter, both *Prochlorococcus* abundance and cell division rates (0.1 to 0.2 day^{-1}) were low. Nutrient concentrations were relatively high, but cell division rates appeared to be limited by irradiance and possibly temperature. In stratified water columns, cell division rates were systematically higher. Cell division rates in the southern Sargasso mixed layer were lower than those in the subtropical Pacific, probably because of more severe nutrient limitation.

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Chapter 5

Summary and Future Directions

The main question addressed in this thesis is what impact do trace metals have on the cell division rates and abundance of cyanobacteria? In the equatorial Pacific the answer to this question has implications for how this ecosystem is regulated. In this region high concentrations of the major nutrients persist in spite of adequate illumination and a stable water column. It has been a matter of debate whether this is the result of high grazing pressure that prevents phytoplankton biomass from reaching the carrying capacity of the major nutrients (Cullen et al. 1992, Frost and Franzen 1992), limitation of cell division rate and productivity by a trace element (Martin et al. 1991), or a combination of both (Frost and Franzen 1992, Price et al. 1994, Landry et al. 1997). Recent results indicate that iron, which is required for chlorophyll synthesis and both photosynthetic and respiratory electron transport, is present in the equatorial Pacific in limiting concentrations (Martin et al. 1994, Coale et al. 1996). When these waters were enriched with iron the abundance of large eukaryotic cells increased by over an order of magnitude (Coale et al. 1996, Cavender-Bares et al. 1998). The photosynthetic efficiency of the phytoplankton community as a whole increased (Greene et al. 1994, Kolber et al. 1994, Lindley et al. 1995, Behrenfeld et al. 1996, Kudela and Chavez 1996) as did the FALS and chlorophyll fluorescence per cell of the marine cyanobacteria *Prochlorococcus* cell size (Cavender-Bares et al. 1998). *Prochlorococcus* cell division rates doubled inside the iron enriched patch and reached two divisions per day in bottle incubations with additional iron (Mann and Chisholm in press, Chapter 2) indicating that these

cells were iron limited. However, *Prochlorococcus* abundance remained constant (Cavender-Bares et al. 1998) because mortality rates increased as fast as the cell division rates (Mann and Chisholm in press, Chapter 2).

One question that remains unanswered is the spatial and temporal variability of *Prochlorococcus* cell division rates. Cell division rates measured here are similar to others measured in the equatorial Pacific (Vaulot et al. 1995, Vaulot et al. 1999). However, primary productivity in the equatorial Pacific can vary depending on the local conditions (Cullen 1995) and *Prochlorococcus* cell division rates may increase during episodic events that increase the availability of iron. For instance, chlorophyll specific productivity was higher when the passage of a tropical instability wave enhanced the upwelling of iron rich water from the Equatorial Undercurrent (Barber et al. 1996). *Prochlorococcus* cell division rate may have increased slightly at the surface at the same time (Vaulot et al. 1995). It would be very interesting to determine if *Prochlorococcus* cell division rates were higher in the iron rich plume downstream from the Galapagos Islands, where both iron and chlorophyll concentrations are at least double those in the surrounding water (Martin et al. 1994).

Prochlorococcus are well adapted to an oligotrophic environment. Their small size increases the relative surface area to volume ratio and makes diffusion limitation by

iron or other nutrients less likely than in larger cells (Morel et al. 1991). While this is advantageous when nutrients or iron are in short supply, a high surface area to volume ratio may increase their exposure to toxic metals as well. The flux of iron to surface waters from atmospheric deposition is about 10 fold higher in the Atlantic than in the Pacific (Duce and Tindale 1991). This makes iron limitation in the Sargasso Sea unlikely, but increases the concentration of other metals in the surface waters. As a result, free Cu^{2+} concentrations in the Sargasso Sea can reach up to 6 pM in shallow mixed layers while those in the subtropical Pacific are at least 30 fold lower (Coale and Bruland 1990, Moffett 1995).

Copper is an essential trace metal, but it is toxic to *Prochlorococcus* isolates in concentrations found in the Sargasso Sea. All of these isolates were more sensitive to copper than *Synechococcus*, but members of the low chl B/A (high light adapted) ecotype were less sensitive than strains with high chl B/A ratios (low light adapted) (Moore et al. 1998, Moore and Chisholm 1999, Chapter 3). In the field there is a rough inverse correlation between *Prochlorococcus* abundance and free Cu^{2+}. When the water column is stratified, free Cu^{2+} concentrations are high in the mixed layer and most of the *Prochlorococcus* population is located below the thermocline where free Cu^{2+} concentrations are lower (Moffett et al. 1990, Olson et al. 1990, Moffett 1995, Chapter 3). The distribution of *Synechococcus* is more uniform with depth(Olson et al. 1990, Chapter 3). The *in situ* concentration of free Cu^{2+} had a

strong effect on the copper sensitivity of *Prochlorococcus*. Net growth rates were substantially reduced when *Prochlorococcus* from environments where the *in situ* free Cu^{2+} was low (deep mixed layers and below the thermocline in stratified water) were exposed to copper. *Prochlorococcus* in shallow mixed layers where *in situ* Cu^{2+} was high were less sensitive to copper and may have been members of the copper resistant low chl B/A ecotype (Chapter 3). *Synechococcus* were relatively copper resistant across a range of environments (Chapter 3). These data are consistent with the hypothesis that ambient copper levels may influence the relative abundance of *Prochlorococcus* and *Synechococcus* in the Sargasso Sea.

One direction for future work is to determine the molecular basis for the differences in copper sensitivity between *Prochlorococcus* ecotypes and *Prochlorococcus* and *Synechococcus*. For instance, one possible reason for the greater copper tolerance of *Synechococcus* could be the production of the strong Cu binding chelator L_1 by these cells (Moffett and Brand 1996), which also synthesize siderophores (iron chelators) (Wilhelm and Trick 1994). Preliminary data indicates that *Prochlorococcus* makes a similar Cu chelator, but this work was done in a non-axenic culture and should be repeated now that bacteria free isolates are available (Moffett, unpub. data). It would also be valuable to know if *Prochlorococcus* can synthesize siderophores. This is likely since *Prochlorococcus* can be iron limited and there is evidence that these cells can produce a Co binding compound (Saito et al. 2000). Other strategies used

by bacteria to gain copper tolerance include copper efflux mechanisms such as Cu

ATPases and reduced copper influx (Cooksey 1993, Brown et al. 1994, Cervantes

and Gutierrez-Corona 1994). Determining net Cu^{2+} influx and efflux using ^{64}Cu in

copper resistant and sensitive cyanobacteria could determine if one of these

strategies was used. The genetic code of the *Prochlorococcus* isolate Med 4, which

has recently become available, could also be screened for sequences matching those

of know metal ATPases.

Prochlorococcus cell division rates in the shallow mixed layers of the northern

Sargasso Sea was not conclusively determined in this study (Chapter 4). It is

probable that cell division rates were lower here than in deeper water, since they are

often reduced close to the surface in the Pacific (Vaulot et al. 1995, Liu et al. 1997,

Vaulot and Dominique 1999) where cells are exposed only to high irradiance and not

high free Cu^{2+} concentrations as well. However, the sharp gradient in

Prochlorococcus number between the seasonal mixed layer and just below it made it

difficult to determine the cell division rates because the shoaling of the thermocline

injected cells from a very different environment into the surface waters. Staining

Prochlorococcus with the DNA stain Heochst, which can more easily distinguish

between heterotrophic bacteria and cyanobacteria, may help in the future. Another

way to expand on this work would be to take samples over a longer time period to

determine how much temporal variability was present. Finally, it would also be

valuable to combine short term cell viability studies with the determination of cell

division rates. In one example, samples could be stained with fluorescein diacetate

(Molecular Probes), which is cleaved inside metabolically active cells into a

fluorescent product.

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Differential response of equatorial Pacific phytoplankton to iron fertilization

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Abstract

Recent unenclosed iron-fertilization experiments in the equatorial Pacific Ocean have shown that phytoplankton biomass can be increased substantially by the addition of iron. Analyses of size-fractionated chlorophyll indicate that much of the increase during the most recent fertilization experiment, IronEx II, occurred in the >10-μm size fraction. We used flow cytometry, combined with taxon-specific pigment measurements by high-performance liquid chromatography (HPLC), to analyze the responses of five different groups of phytoplankton: *Prochlorococcus, Synechococcus,* ultraplankton, nanoplankton, and pennate diatoms. These results are unique in the suite of measurements from the IronEx studies in that they simultaneously examine individual cell properties, which are grazer independent, and population dynamics, which reflect the net result of growth and grazing. Our results show that the overall increase of chlorophyll *a* (Chl *a*) in the patch was due in part to increases in chlorophyll content per cell and in part to increases in cell numbers of specific groups. Cellular fluorescence was stimulated by iron addition in all five groups to a qualitatively similar degree and was correlated with taxon-specific changes in cellular pigments. In terms of net cell growth, however, these groups responded very differently. The groups that dominated the community before the addition of iron increased at most twofold in cell number; *Prochlorococcus* actually decreased. In contrast, the initially rare pennate diatoms increased 15-fold in number by the peak of the iron-induced bloom. Within 1 week, this differential response led to a dramatic change in the phytoplankton community structure, from one dominated by picoplankton to one dominated by large diatoms. It is not known whether this shift would be sustained over extended periods of fertilization, a response that would ultimately change the structure of the food web.

Oceanographers have long been puzzled by the simultaneous abundance of macronutrients, such as nitrate and

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phosphate, and the paucity of phytoplankton biomass in the three high-nutrient, low-chlorophyll (HNLC) regions of the world ocean: the equatorial Pacific, the subarctic Pacific, and the Southern Ocean. Early oceanographers noted these anomalies (Gran 1931; Hart 1934), and many hypotheses have been offered and debated over the years (*see* Chisholm and Morel 1991), the most compelling of which are the "iron hypothesis" (Martin 1990), the "grazing hypothesis" (Walsh 1976; Frost 1991), and the unifying "ecumenical hypothesis" (Morel et al. 1991a; Price et al. 1994). The debate on the iron hypothesis, based largely on iron-enriched "grow out" experiments (e.g., Martin and Fitzwater 1988; Buma et al. 1991; Coale 1991; Takeda and Obata 1995; Boyd et al.

1996; Coale et al. 1996*a;* Zettler et al. 1996), culminated with two open-ocean in situ iron-fertilization experiments (IronEx I and II; Martin et al. 1994; Coale et al. 1996*b*).

The biological response to added iron during IronEx II, as indicated by changes in Chl *a,* exceeded the observed increase in IronEx I by nearly one order of magnitude (Martin et al. 1994; Coale et al. 1996*b*). A large portion of the response to iron fertilization was due to a bloom of initially rare diatoms, whose biomass increased 85-fold inside the iron-fertilized patch (Coale et al. 1996*b*). Concomitant with this massive increase in phytoplankton biomass was a drawdown of roughly 5 μM nitrate, attributed almost completely to cells >5 μm (Coale et al. 1996*b*). Other changes of biogeochemical significance included a decrease in CO_2 fugacity of nearly 100 μatm (Cooper et al. 1996), a 3.5-fold increase in dimethyl sulfide production (DMS; Turner et al. 1996), and a 7‰ enrichment in the δ ^{13}C of phytoplankton (Bidigare et al. submitted).

There is evidence that the smaller, initially dominant phytoplankton were also stimulated by the iron addition. Using epifluorescence microscopy, Coale et al. (1996*b*) found modest increases in cell biomass for the smaller, nondiatom taxonomic groups. This analysis did not include the ubiquitous picoplankter *Prochlorococcus,* which cannot be resolved easily using this method. Photochemical quantum efficiency, the ratio of the quantum yield of variable fluorescence (F_v) to that of the light-saturated fluorescence yield (F_m), increased dramatically in both experiments hours after the addition of iron (Kolber et al. 1994; Behrenfeld et al. 1996). Behrenfeld et al. (1996) contend that since F_v/F_m increased prior to a shift in species composition, all phytoplankton taxa were physiologically limited by iron—reinforcing the finding of significant increases in F_v/F_m for all size fractions during IronEx I (Kolber et al. 1994). In addition, results from bottle dilution experiments (Landry and Hassett 1982) indicate that the net autotrophic growth rate of the phytoplankton community <5 μm in size more than doubled inside the enriched patch while the microzooplankton grazing rates increased threefold (Coale et al. 1996*b*). Higher grazing rates inside the patch and only a modest increase in small phytoplankton biomass suggest that all phytoplankton were stimulated by the addition of iron, but only the initially rare diatoms escaped grazing pressure and bloomed.

Most of the reports to date use bulk measurements, which do not distinguish between subgroups, to investigate phytoplankton dynamics inside the iron-fertilized patch. We used analyses of size-fractionated chlorophyll to monitor changes between large (>10 μm) and small (<10 μm) size classes following enrichment. To follow the response of specific groups of phytoplankton to iron enrichment, we used flow cytometry, combined with taxon-specific pigment measurements. This allowed us to distinguish between changes in cell number and cellular chlorophyll since a change in either would affect bulk chlorophyll measurements. Moreover, flow cytometric analyses provide a measure of cell-specific responses to iron enrichment, independent of any changes, or lack thereof, in population numbers. Finally, this .is the first report documenting the response to in situ iron fertilization of the picophytoplankter *Prochlorococcus,*

which is typically a large contributor to standing stocks in the equatorial Pacific (e.g., Binder et al. 1996).

Methods

Iron patch and sampling—IronEx II was conducted between 29 May and 15 June 1995 starting at 4°S, 105°W in a region with high (ca. 10 μM) nitrate concentrations (Coale et al. 1996*b*). Iron, added as Fe(II) in seawater at pH 2, was injected into surface waters three times over a 7-d period, initially forming a 72-km^2 patch. The target iron concentration for the initial fertilization was 2 nM—a 40-fold increase from the 0.05-nM ambient concentration; the second and third injections were designed to add another 1 nM Fe each (Coale et al. 1996*b*). A 1,000-km^2 survey of the general study site was conducted prior to the first iron infusion to ensure against possible patch subduction, as occurred in IronEx I (Martin et al. 1994), and to verify that a representative site was chosen. Stations were occupied daily inside and outside the patch, although in some cases, only one of these was possible. All data presented here are from stations sampled at local dawn to eliminate complications arising from diel patterns.

Pigments—We used size-fractionated Chl *a* concentrations to compare broad changes in phytoplankton community structure in and out of the patch. The fractions were analyzed in duplicate by parallel filter fractionation, using polycarbonate 10-μm pore-size filters (Poretics) and Whatman GF/F filters for the total. After extraction in 90% acetone for 24 h, Chl *a* was assayed fluorometrically (Parsons et al. 1984) on a 10-AU fluorometer (Turner Designs), which was calibrated using extracts of spinach Chl *a* (Sigma-Aldrich). Chl *a* in the >10-μm fraction equaled the material retained on the 10-μm filter, whereas the <10-μm fraction was the difference between the total and that retained on the 10-μm filter.

Taxon-specific pigments were monitored in whole-water (bulk) samples to complement the flow cytometry data (*see below*). Seawater samples were filtered onto Whatman GF/F filters, and acetone extracts were then analyzed by HPLC for chlorophyll and carotenoid quantification (Goericke and Repeta 1993). Divinyl chlorophyll *a* (Chl a_2) and fucoxanthin were used as chemotaxonomic markers for *Prochlorococcus* spp. and diatoms, respectively. The sum of 19′-hexanoyloxyfucoxanthin, 19′-butanoyloxyfucoxanthin, and peridinin (HBP) was used as a marker for the group consisting of pelagophytes, prymnesiophytes, and dinoflagellates (Vesk and Jeffrey 1987; Wright and Jeffrey 1987; Simon et al. 1994; Andersen et al. 1996). It has been shown that prymnesiophytes and dinoflagellates contribute significantly to the nanophytoplankton (2–20 μm) biomass of the equatorial Pacific (Chavez et al. 1990; Coale et al. 1996*b*), and it is likely that the pelagophytes are in the group of eukaryotic picoplankton (<2 μm) termed the "red fluorescing picoplankton" (Chavez et al. 1990; Coale et al. 1996*b*). Thus, for the sake of comparison, we have used the HBP sum to represent the pigment biomass of the ultra- and nanophytoplankton groups combined (*see below*). Note that chemotaxonomic markers for *Synechococcus* were not measured.

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Flow cytometry—A modified Coulter EPICS V flow cytometer (Cavender-Bares et al. 1998) was used to characterize the phytoplankton community in terms of autofluorescence per cell, which is related to pigment concentration (Sosik et al. 1989; Li et al. 1993; Jonker et al. 1995), and forward angle light scatter (FALS) per cell, which is correlated with cell size (Van de Hulst 1957; Ackelson and Spinrad 1988). A 40.5-mm spherical lens was used to focus the 488-nm laser beam (Innova 90 at 800 mW; Coherent), either alone for picoplankton analyses or in combination with a 150-mm cylindrical lens, which was focused on the back focal plane of the spherical lens, for analyses of larger cells. These configurations produced a 17-μm circular and a 17- $\times$ 400-μm elliptical laser spot, respectively, for sample illumination within the quartz flow cell. Chlorophyll or red fluorescence (Red FL) signals passed first through a 488-nm long-pass filter, then were reflected by a 630-nm short-pass dichroic filter, and finally passed through a 680-nm bandpass filter (40-nm bandwidth; Omega Optical). Phycoerythrin or orange fluorescence (Orange FL) signals passed through the 630-nm short-pass dichroic and also through a 515-nm long-pass filter. FALS signals, which passed beyond a wide obscuration bar and then through a vertical polarization filter, were measured using a PMT (photo multiplier tube) detector. Log-integrated values were collected for all three signals. When the range of fluorescence values exceeded the nominal three-decade range of the instrument, we employed an extra electronic circuit to expand the Red FL scale. A subtraction module can be used to subtract electronically one signal from a second and is typically used to separate spillover of one signal into a second. Only partial subtraction of one signal from the other is possible with a maximum of 50% for those modules associated with the flow cytometer. Two modules were used in series, and the Red FL signal from the PMT was split and input as two separate signals, resulting in a new signal that equaled 25% of the original Red FL signal. This "reduced" Red FL signal was used to characterize those cells that were offscale on the standard Red FL scale.

We are able to differentiate five separate groups of photosynthetic cells based on their distinct fluorescence and light scatter signals as represented in Fig. 1. The two cyanobacteria *Prochlorococcus* and *Synechococcus* are easily differentiated from each other since only *Synechococcus* exhibits Orange FL (Chisholm et al. 1988; Olson et al. 1990). Both cyanobacteria have relatively low FALS signals, which separates them from the ultra- and nanoplankton. The smallest ultraplankton are also distinguishable from *Synechococcus* because of their lack of Orange FL. The ultra- and nanophytoplankton are identified based on their size and fluorescence characteristics (Zettler et al. 1996), and pennate diatoms can be identified because they scatter less light than would be expected for their size because of their long, slender shape (Olson et al. 1989; Zettler et al. 1996). Fluorescence and light scatter values are reported relative to the value of calibration microspheres added to each sample (Polysciences; 0.474 and 2.02 μm [Fig. 1A,B and C, respectively]). Note that although red fluorescence values are shown in Fig. 1 for *Synechococcus,* they are not reported in our
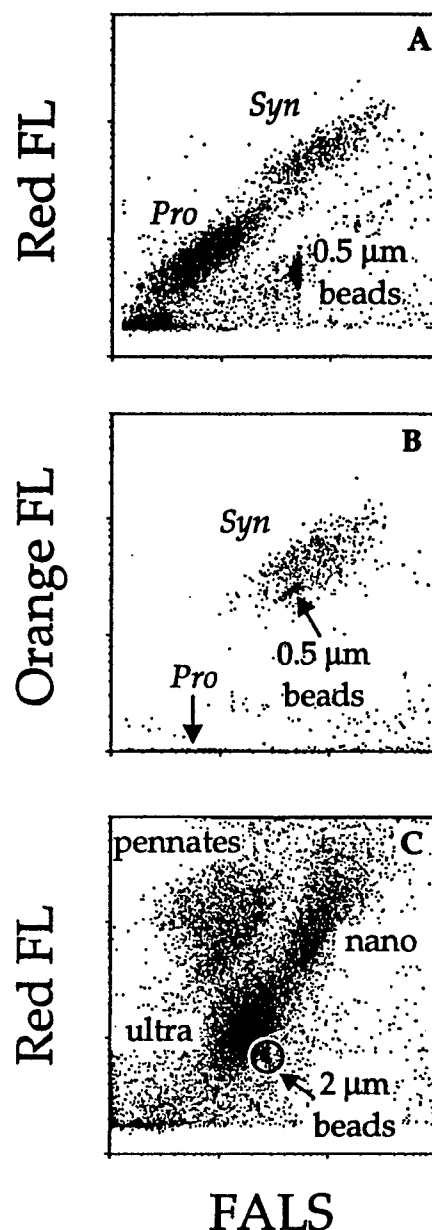
Fig. 1. (A) Flow cytometric scattergram of *Prochlorococcus* and *Synechococcus,* with Red FL plotted against FALS. (B) Orange FL vs. FALS scattergram showing how *Synechococcus* are separated from *Prochlorococcus,* since the latter do not exhibit Orange FL. (C) Scattergram showing the larger cells in the ultra- and nanoplankton and pennate diatom groups. Each dot denotes an individual cell for a 10-m sample taken on day 7 inside the iron patch. Calibration microspheres (beads) of different sizes were added to each sample as internal standards: 0.47 μm for A,B; and 2.02 μm circled in C.

results since a portion of the orange fluorescence from phycoerythrin may also have been detected as red fluorescence.

To put bounds on the sizes of the flow cytometrically determined ultra- and nanoplankton groups, we analyzed material that passed through differently sized Poretics polycarbonate membrane filters. On day 5 inside the patch, 75% of the ultraplankton passed through a 2-μm filter, while 95% passed through a 5-μm filter. On the same day, 50% of the nanoplankton passed through a 2-μm filter, and >95% passed through a 10-μm filter. These flow cytometric populations do not conform well to the "pico" (<2 μm) and "nano" (2–20 μm) size ranges as originally defined (Sieburth et al. 1978); therefore, our use of "ultra" and "nano" modifiers in this paper is operational. Determining that 60% of the pennates passed through a 10-μm filter reveals little about their dimensions because of their long slender shape and uncertainty as to how they would pass through a filter. However, Zettler et al. (1996) found that this flow cytometric group consisted mainly of cells up to 50 μm in length, in agreement with microscopic observations during IronEx II, which found this group included cells up to roughly 60 μm in length (Tanner pers. comm.).

There is good evidence that flow cytometrically derived fluorescence per cell is related to pigment per cell for individual species (e.g., Sosik et al. 1989; Li et al. 1993; Jonker et al. 1995; Moore et al. 1995; Moore and Chisholm in press), and we demonstrate this directly for *Prochlorococcus* in this study (*see below*). This relationship need not be linear, however, and can be confounded by variations in accessory pigments and the "package effect" (Sosik et al. 1989). Thus, particularly for larger cells, equivalent increases in pigment per cell may not result in proportional increases in Red FL per cell, thereby limiting quantitative interspecific comparisons of cellular fluorescence. The relationship between cellular fluorescence and absolute pigment concentration may also be influenced by changes in fluorescence yield (Falkowski and Kiefer 1985; Falkowski and Kolber 1995). Since the addition of DCMU [3-(3, 4-dichlorophenyl)−1,1-dimethylurea] does not affect flow cytometrically derived fluorescence signals except at excitation intensities well below those used in this study (Xu et al. 1990; Furuya and Li 1992), we do not consider variable fluorescence yield to be an issue in interpreting our data.

Results and discussion

Size-fractionated Chl a—Total Chl *a*, a proxy for phytoplankton biomass, averaged 0.2 μg liter^{-1} in surface waters outside the iron-enriched patch (Fig. 2A), a typical value for the equatorial Pacific (Chavez et al. 1990). After 2 d of iron enrichment, total Chl *a* had doubled, and by day 6, it had increased 12-fold to 2.6 μg liter^{-1} (Fig. 2A,B). Initially, the phytoplankton community was dominated by cells <10 μm, which accounted for 90% of the total. By day 6, total chlorophyll in the <10- and >10-μm fractions was roughly equal inside the patch and had increased 7- and 60-fold, respectively (Fig. 2A,B). The >10-μm fraction rose from 10 to 60% of the total, representing a major shift in community composition from small to large cells (Fig. 2C). This shift
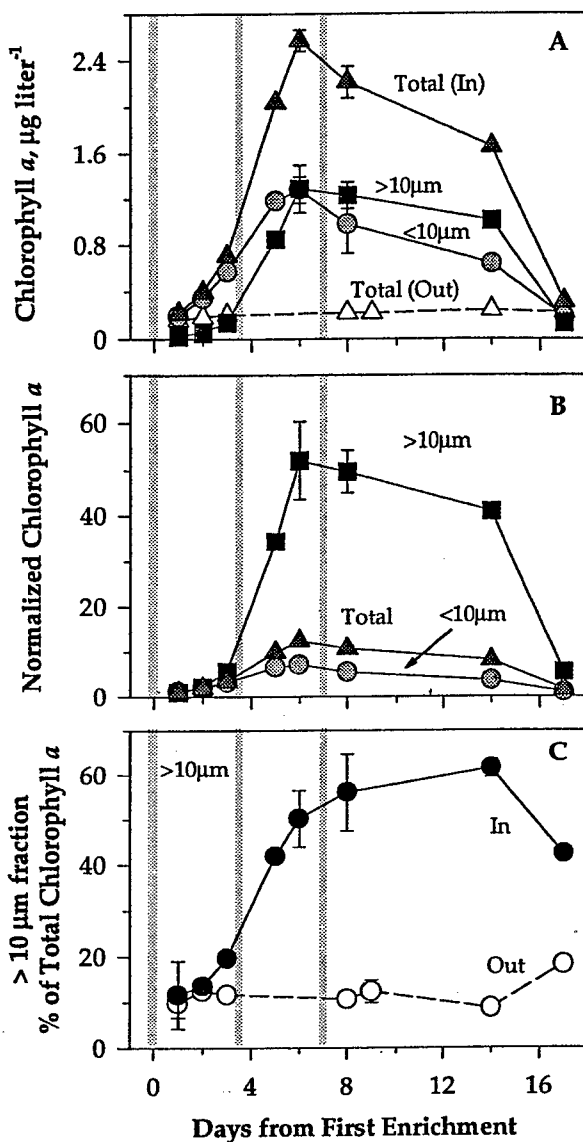
Fig. 2. Size-fractionated Chl *a* ± 1 SD at 15 m as a function of time over the course of the iron-enrichment experiment. (A) Concentrations inside the iron-enriched patch (solid symbols and lines) and outside the patch (open symbols, dashed line). (B) Concentrations measured inside the patch normalized to the average for each fraction outside the patch. (C) The >10-μm fraction is shown as a percentage of the total Chl *a* inside and outside the patch (symbols and lines as in A). Days of iron addition are marked with vertical bands.

is consistent with observations that high total Chl *a* is accompanied by an abundance of large cells (Raimbault et al. 1988; Chavez et al. 1996). Judging from the analysis of material passing through filters (discussed in Methods), all of the flow cytometrically defined groups contributed to the

<10-μm fraction, while a significant portion of the pennate diatoms were caught on 10-μm filters, thereby contributing to the >10-μm fraction.

Flow cytometric pigments—Chlorophyll fluorescence (or in the case of *Synechococcus*, phycoerythrin fluorescence) per cell increased in all groups over the first 7 d of the experiment (Fig. 3A). The simplest interpretation of these increases, supported reasonably well by the taxon-specific pigment data presented below, is that they reflect an increase in cellular pigment concentration for all groups in response to iron enrichment, which indicates that all of these groups were iron limited at the start of the experiment. Such an interpretation is consistent with what is known about the physiological responses of phytoplankton when released from nutrient limitation, since increased cellular Chl a concentration is a standard response when phytoplankton are shifted from nutrient-limited to nutrient-replete conditions (e.g., Eppley and Renger 1974; Perry 1976). This pattern has held true in a range of iron-limited phytoplankton cultures (Geider et al. 1993; Trick et al. 1995; Wilhelm and Trick 1995; Kudo and Harrison 1997; McKay et al. 1997; Sunda and Huntsman 1997) and can be indicative of the degree of iron limitation. However, because of the potentially complex relationship between flow cytometrically derived cellular fluorescence and pigment per cell, we cannot interpret the relative magnitude of the fluorescence response between the groups as a measure of the degree of iron limitation. Further, a change in species composition within a group over time might influence its mean cellular fluorescence—a nuance that would be missed by the process of averaging. That is, it is impossible to distinguish between either an entire group, or just a subset of that group responding to iron, if there is no change in shape of the flow cytometric signature.

Taxonomic marker pigments by HPLC—Pigments that are indicative of different classes of phytoplankton were measured over the course of the experiment using HPLC. By normalizing these pigment concentrations to the cell numbers in the different groups defined flow cytometrically, we used these data to interpret Red FL cell^{-1} values and to help judge how well the pennate diatoms detected by flow cytometry represented the larger diatom community as a whole. Cellular Red FL values are expected to covary with xanthophyll concentrations for the ultra- and nanoplankton and pennate diatom groups, since they absorb light at the excitation wavelength used in the flow cytometer (488 nm) and pass energy on to Chl a (Bidigare et al. 1990). In contrast, Chl a_2 was directly compared to cellular Red FL for *Prochlorococcus*, as it is unique to this group. When plotted on a relative scale, we found that variations in cell-specific pigment concentrations determined for *Prochlorococcus* and the ultra- and nanophytoplankton combined (*see Methods*) followed the same patterns as those observed for the corresponding cellular Red FL values (Fig. 4A,B). Calculated cellular Chl a_2 cell^{-1} for *Prochlorococcus* ranged from 0.2 to 0.9 fg cell^{-1}, which is consistent with values measured in laboratory cultures (Moore et al. 1995; Moore and Chisholm in press). Because *Prochlorococcus* uniquely contains Chl

a_2, the significant correlation shown in Fig. 4D indicates that when Chl a_2 cell^{-1} doubled, so did cellular Red FL (model II regression; Sokal and Rohlf 1995). For the ultra- and nanoplankton, when HBP cell^{-1} doubled, so did Red FL cell^{-1} (Fig. 4E). Yet, without knowing the relationship between cellular HBP and Chl a, a strong conclusion about the relationship between Red FL cell^{-1} and Chl a cell^{-1} cannot be made for this group. A varying cellular HBP:Chl a ratio would, for instance, likely cause variation in the relationship between cellular Red FL and Chl a (Sosik et al. 1989).

To estimate the degree to which the total diatom community can be represented by our flow cytometric pennate diatom group, we compared the relative changes in the bulk concentration of the diatom-specific pigment fucoxanthin with the relative changes in total pennate fluorescence measured per liter (Fig. 4C,F). Diatoms as a group were represented by more than the pennates over the course of the experiment (Coale et al. 1996b). The largest diatoms, particularly the chain-forming types, would have been poorly sampled by flow cytometry. In addition, a second pennate diatom population, common to iron-enrichment studies (Zettler et al. 1996), was detected on days 6–8 only. This population was offscale on the y-axis above the pennate population depicted in Fig. 1C and had a low cell abundance (maximum = 450 cells ml^{-1}), but it had a 10-fold higher mean Red FL cell^{-1} than the more numerous pennate population. This ephemeral population was not represented in Fig. 3 because the cells were undetectable outside the patch, but we do include it in the bulk Red FL data for Fig. 4. The difference between the dashed and solid half-tone lines in Fig. 4C indicates the importance of this population to the total Red FL per milliliter. By day 8, this second pennate population had waned, and our subsequent flow cytometric analyses clearly missed a large component of the diatom community, as is evidenced by the disparity between the bulk concentrations of Red FL and fucoxanthin (Fig. 4C). For this reason, data from day 8 were excluded from the regression in Fig. 4F. However, the agreement between pigment and fluorescence concentrations prior to day 8 (Fig. 4C) confirms that the pennate diatoms we identified by flow cytometry were the major contributors to the total diatom assemblage up to that point.

Individual cell size—Mean FALS per cell, an indicator of cell size (Van de Hulst 1957; Ackelson and Spinrad 1988), increased over the first 7 d of the experiment for all groups, roughly paralleling changes in fluorescence per cell (Fig. 3B). Mean FALS per cell increased more than twofold for *Prochlorococcus* and *Synechococcus*, which corresponds approximately to a 1.5-fold increase in cell volume for these species. Similar increases for the ultra- and nanophytoplankton over this same period reflect roughly a twofold increase in mean cell volume (data not shown; *see* DuRand 1995; Dusenberry 1995). The mean FALS per cell for the pennate diatoms, which could not be calibrated because of the geometry of the cells (Olson et al. 1989), showed an increase similar to that of the other populations, but not as pronounced.

Given the simultaneous increase in cellular FALS and FL in all populations, it is unclear if the response to iron en-

A
Normalized
mean FL cell⁻¹

B
Normalized
mean FALS cell⁻¹

C
Normalized
Concentration

Days from First Enrichment
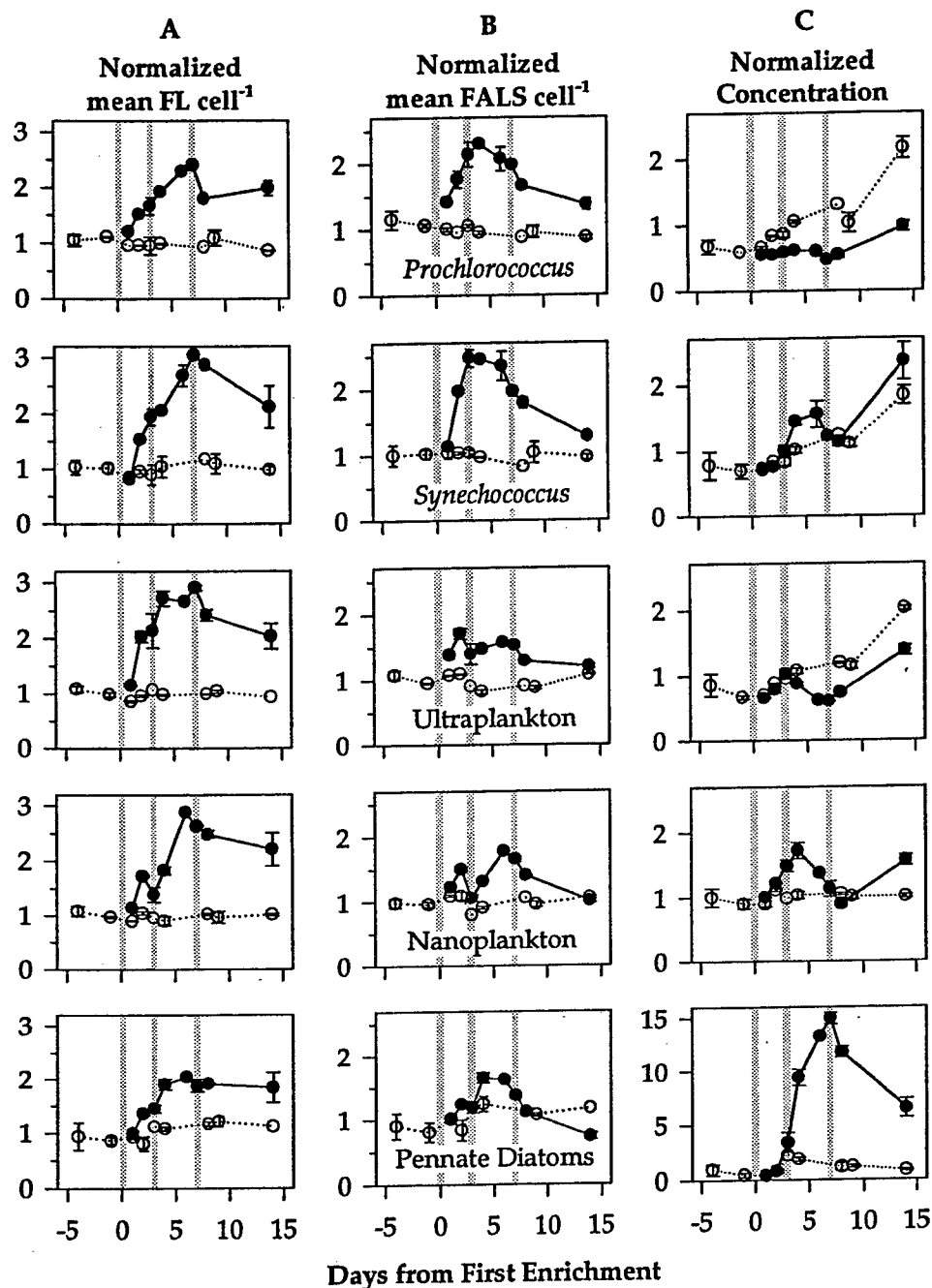
Fig. 3 Response of different phytoplankton groups to iron enrichment in the patch (solid symbols and lines) relative to outside the patch (open symbols, dashed lines) as measured by flow cytometric analysis of individual cells. (A) Normalized mean pigment fluorescence per cell: Orange FL for *Synechococcus* and Red FL for the other groups; (B) normalized mean FALS per cell; and (C) normalized cell concentration. All values reported normalized to the average values outside the patch; data points typically represent average mixed-layer values (3, 10, and 15 m ± 1 SD). Average cell concentrations outside the patch were 1.4×10^5, 7.6×10^3, 1.1×10^4, 2.0×10^3, and 1.4×10^2 cells ml⁻¹ for *Prochlorococcus, Synechococcus,* ultraplankton, nanoplankton, and pennate diatoms, respectively. Note change of scale in normalized concentration for the pennate diatoms. Days of iron addition are marked by vertical bands.
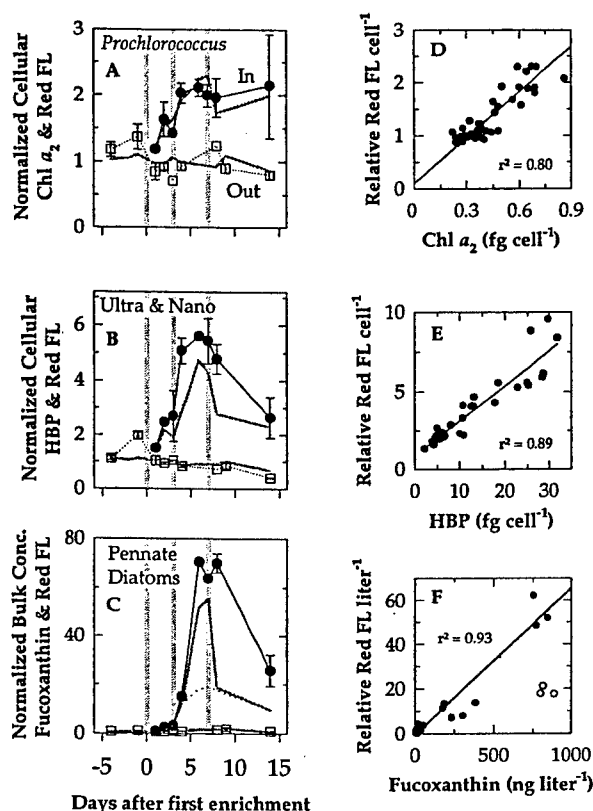
Fig. 4 (A–C) Phytoplankton pigments in the patch (solid circles and lines) and outside the patch (open squares, dashed lines) as measured by HPLC, with corresponding flow cytometry data from Fig. 3 shown for reference (half-tone lines). (A) Normalized cell-specific Chl a_2 concentration and Red FL for *Prochlorococcus*. (B) Normalized cell-specific HBP concentration and Red FL for the ultra- and nanoplankton combined. In A and B, bulk pigment concentrations representative of each of the groups of phytoplankton were divided by the flow cytometrically derived cell abundances to yield pigment per cell; they were then normalized to the average value of 3-, 10-, and 15-m samples outside of the patch (± 1 SD), as in Fig. 3. (C) Normalized bulk concentrations of fucoxanthin and red fluorescence (Red FL cell^{-1} $\times$ cells liter^{-1}); a second dashed half-tone line added to show the contribution of the numerically dominant pennate population, whereas the solid half-tone line combines red fluorescence values from this and the far less abundant, but highly pigmented, pennate population that appeared on days 6–8 (*see text*). (D–F) Relationship between HPLC and flow cytometry data using all of the non-normalized data from (A–C), respectively, including Model II regression lines (reduced major axis; Sokal and Rohlf 1995). Note that the data from day 8 (open circles) were excluded from the regression in F.

richment involved an increase in the volumetric concentration of pigment or whether pigment simply increased in proportion to increases in cellular volume. For all the groups except *Prochlorococcus*, the uncertainties involved in converting FL to Chl *a* and FALS to volume are too large to distinguish between these two possibilities. Based on culture

studies (Moore et al. 1995; Moore and Chisholm in press) and the data reported here, we know that the 2.5-fold increase in cellular Red FL for *Prochlorococcus* over the first 4 d of the experiment (Fig. 3A) corresponded to a 2.5-fold increase in Chl a_2 cell^{-1} (Fig. 4D). Using an empirical calibration of FALS to volume for this species (data not shown) and evidence from synchronized populations in cultures and in the field, where the drop in the FALS signal as cells divide reflects a halving of the cell volume (DuRand 1995; Dusenberry 1995; Binder et al. 1996), we find that over the first 4 d, the 2.3-fold increase in mean FALS per cell for *Prochlorococcus* (Fig. 3B) was associated with a 1.6-fold increase in mean cell volume. Thus, these data suggest that Chl a_2: volume for *Prochlorococcus* increased approximately 1.5-fold inside the patch. That is, *Prochlorococcus* increased Chl a_2 beyond that which would have been necessary simply to match increases in cell volume. An elevated Chl a_2: volume is consistent with a positive correlation between Chl a:C and iron concentration observed in iron-limited cultures of coastal eukaryotic phytoplankton (Sunda and Huntsman 1997) since cell volume and cell C are positively correlated (Strathman 1967; Lee and Fuhrman 1987; Simon and Azam 1989; Verity et al. 1992). We are currently working to constrain the uncertainty in estimates of Chl a: volume for the other phytoplankton groups based on these flow cytometric data.

Cell number response—In terms of cellular Red FL and FALS, all of the groups responded to iron enrichment in a qualitatively similar manner. However, when changes in cell numbers are considered, dramatic differences between the groups are apparent (Fig. 3C). The pennate diatoms, which averaged 140 cells ml^{-1} outside the patch, reached 2,100 cells ml^{-1} inside the patch. Over the first 4 d, their net growth (reflecting μ, the rate of cell division, minus cell losses) was exponential, and they had a net growth rate (μ_{net}) of 1.0 d^{-1}. There was a distinct increase in ultraplankton cell numbers inside the patch, but a similar increase was observed outside the patch; thus, the former cannot necessarily be attributed to iron addition. The nanoplankton, however, displayed increases in cell number inside the patch by day 3, more than doubled in number by day 4, and then returned to out-of-patch levels. In contrast, the increase in cell numbers of the smaller phytoplankton inside the enriched patch was modest at best. Relative to unfertilized waters, *Prochlorococcus* cell numbers actually decreased inside the patch. Using cell cycle analysis, Mann and Chisholm (submitted) found that μ for *Prochlorococcus* increased approximately 60% inside the patch, which suggests that the decrease in *Prochlorococcus* cells inside the patch was due to increased grazing rates.

Synechococcus cell numbers increased twofold inside the patch by day 7, but they then quickly receded to match out-of-patch populations. The majority of this increase occurred between days 3 and 4, suggesting a rapid decrease in grazing pressure, an increase in μ, or both. Rue and Bruland (1997) found increased production of an organic ligand capable of binding iron shortly following iron enrichment. *Synechococcus* is known to produce such ligands in culture, apparently to maintain high growth rates at very low iron concentrations

(Wilhelm et al. 1996). Production of siderophores by *Synechococcus* would increase their access to the iron pool and could therefore be viewed as a signal that *Synechococcus* responded to the iron addition much as if it were an aeolian deposition event. Speculating one step further, if the growth rate of *Synechococcus* were limited by iron, sequestration of added iron by newly produced siderophores would enhance the availability of iron to *Synechococcus*, leading to an increase in μ.

These data indicate that even though some of the smaller phytoplankton groups increased twofold in concentration, only the initially rare pennate diatoms broke free from grazer control. This is in agreement with evidence that microzooplankton exert strong control over the typically dominant phytoplankton of the equatorial Pacific (Price et al. 1994; Landry et al. 1995, 1997) and with evidence suggesting that an increased μ of the phytoplankton community was matched by heightened microzooplankton grazing pressure during IronEx II (Coale et al. 1996b). Assuming that cell losses were dominated by the rate of grazing (g) during the early stages of the iron-enriched patch (i.e., $\mu_{net} \approx \mu - g$), then a simple explanation for the bloom of pennates is that iron fertilization caused a substantial elevation in μ relative to g for these populations (Cullen 1995), resulting in the measured μ_{net} of 1.0 d^{-1}. This is in accord with the limited grazing impact of the mesozooplankton measured inside the patch (Coale et al. 1996b). After this growth burst, cell numbers declined, even though there had been a third addition of iron on day 7 and mean fluorescence per cell remained elevated over out-of-patch levels (Fig. 3A).

The decline in pennate cell numbers could have resulted from a combination of events, including increased removal rates, such as grazing and or sinking (Bidigare et al. submitted), or reduction in growth rate because of limitation by another factor. ^{234}Th inventories measured during IronEx II indicate that the particulate organic carbon (POC) flux increased sevenfold between days 7 and 14 following initial enrichment (Bidigare et al. submitted). Kudela and Chavez (1996) suggest that the decreased transparency of patch waters may have induced light limitation of phytoplankton growth. As argued by Erdner (1997), incomplete relief from iron limitation was also a possibility. She showed that there was no detectable switch from flavodoxin to ferredoxin in the phytoplankton community throughout IronEx II, as would be expected upon the relief of iron limitation. In addition, silica, which is essential for diatom growth, has recently been argued as the ultimate factor producing HNLC conditions in the equatorial Pacific (Dugdale et al. 1995; Dugdale and Wilkerson 1998). As nitrate dropped from ca. 10 μM outside the patch to 7 μM inside on day 7 (Coale et al. 1996b), silicate dropped from ca. 4 μM to 1 μM (Coale pers. comm.). Dugdale and Wilkerson (1998) argue that at silicate concentrations below roughly 2 μM, nitrate uptake by diatoms is curtailed, which could help explain the decrease in net growth seen for the pennate diatoms after day 7 (Fig. 3C).

Conclusions

These results document clear differential changes within the phytoplankton community over the course of the iron-

enriched bloom during IronEx II. Using fractionated Chl *a*, we measured a dramatic size-based shift as biomass in the >10-μm fraction increased 60-fold. The overall increase in Chl *a* resulted in part from increases in cellular pigment concentration, which were amplified to varying extents by increases in cell numbers across the groups studied. The pennate diatoms, which increased >10-fold, were the only cells identified in this study that bloomed following iron enrichment. These findings are consistent with the recent equatorial Pacific synopsis (Landry et al. 1997), as well with as the "ecumenical iron hypothesis" (Morel et al. 1991b; Price et al. 1994; Cullen 1995), which allows for the coregulation of phytoplankton in this region by iron limitation and grazing, the latter playing a particularly significant role in regulating the small, dominant cells.

In our view, the most significant conclusion from the IronEx experiments and related work (e.g., Martin and Fitzwater 1988; Coale 1991; Takeda and Obata 1995; Boyd et al. 1996; Coale et al. 1996a; Zettler et al. 1996) is that phytoplankton community structure changes dramatically when productivity is stimulated by nutrient enrichment. As pointed out by Howarth (1988), the concept of "nutrient limitation" must include both the limitation of extant dominant species and the limitation of the potential rate of net primary production, which can only be realized upon the supply of the limiting nutrient together with an accompanying shift in community structure. It is important to keep in mind that the response of the phytoplankton community seen in IronEx II was transient. We do not know what the structure of the food web would look like if iron were supplied continuously over time scales much longer than the generation times of the relevant organisms. Modeling studies (e.g., Sarmiento and Orr 1991; Frost and Franzen 1992; Armstrong 1994), which take into account the potential for changes in community structure, will be important for projecting hypothetical outcomes to fertilization scenarios. Only through carefully designed long-term experiments, however, will we be able to predict the ecological consequences of, and changes in export carbon resulting from, ocean fertilization. These will be important for developing policies to regulate commercial ocean fertilization (Markels 1995; Sørensen 1995; Jones 1996; Jones and Young 1997).

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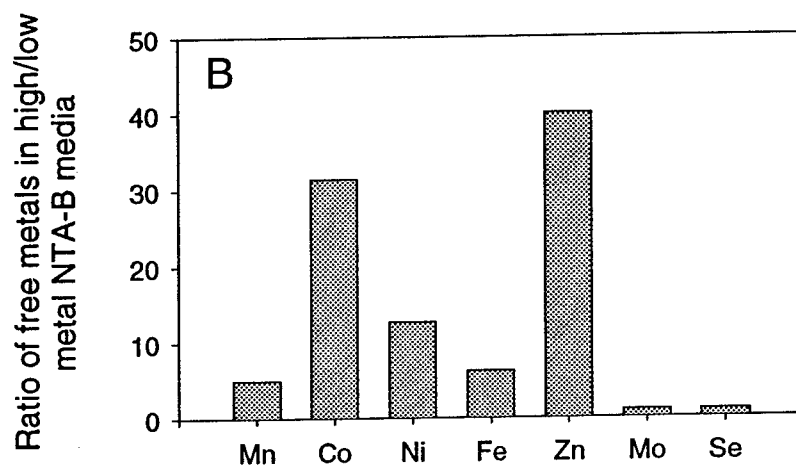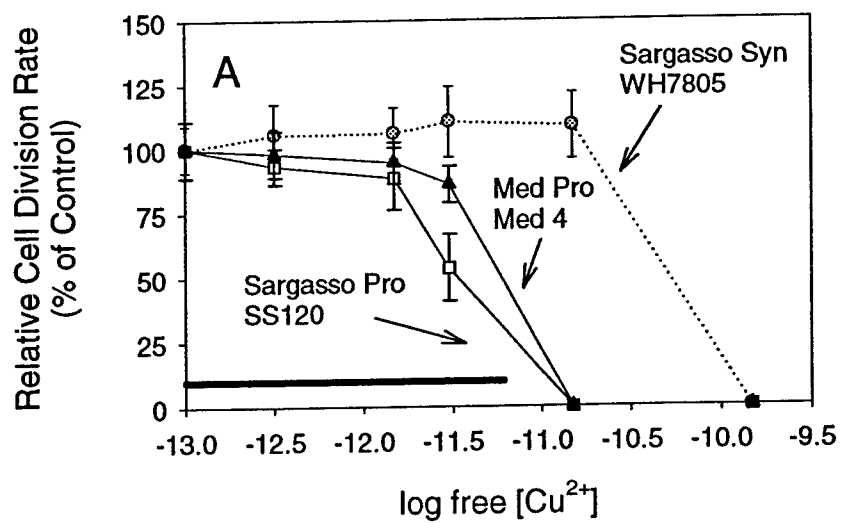
Appendix B

Ancillary Data for Chapter 4

Fig 1A) Cell division rates of *Prochlorococcus* and *Synechococcus* isolates as a function of free Cu^{2+} in a low metal media. The cell division rates are relative to those of the control. Cell division rates in the controls ranged from 0.32 to 0.4 day^{-1}. Light availability and culturing methods were similar to those described in Chapter 3. The dark bar represents the range of free Cu^{2+} expected in the Sargasso Sea. The copper sensitivity of the cyanobacteria was greater than in Chapter 3, Fig 3. For instance *Synechococcus* WH7805 could not grow at a free Cu^{2+} concentration that did not decrease the cell division rate in the earlier data. The concentrations of trace metals in the media used here are lower than those in Chapter 3 (see below). Since trace metal toxicity is dependent on the ratio of toxic to nutrient metals, the result is a greater sensitivity to added copper. Note however that the relative copper sensitivity of the cyanobacteria has not changed compared to the data in Chapter 3; the *Synechococcus* isolate was still the most resistant to copper followed by high light adapted (low chl B/A) *Prochlorococcus* isolate Med 4. The low light adapted (high chl B/A) *Prochlorococcus* isolate SS120 was the most copper sensitive.

Fig 1B) The ratio of free trace metals in the high metal media used in Chapter 3 and the low metal media used in this study. The composition of the low trace metal media is listed below in Table 1 below (compare to Chapter 3, Table 1). Competitive interactions in which a high concentration of one metal reduces the toxicity of another have been found for Cu and Fe, Cu and Mn, and Cu and Zn (Chapter 3 and references therein). In this case several metals were in much higher concentration in the high metal media, so it is difficult to determine what metal was responsible for the changes in relative copper toxicity.

Composition of the low trace metal media.

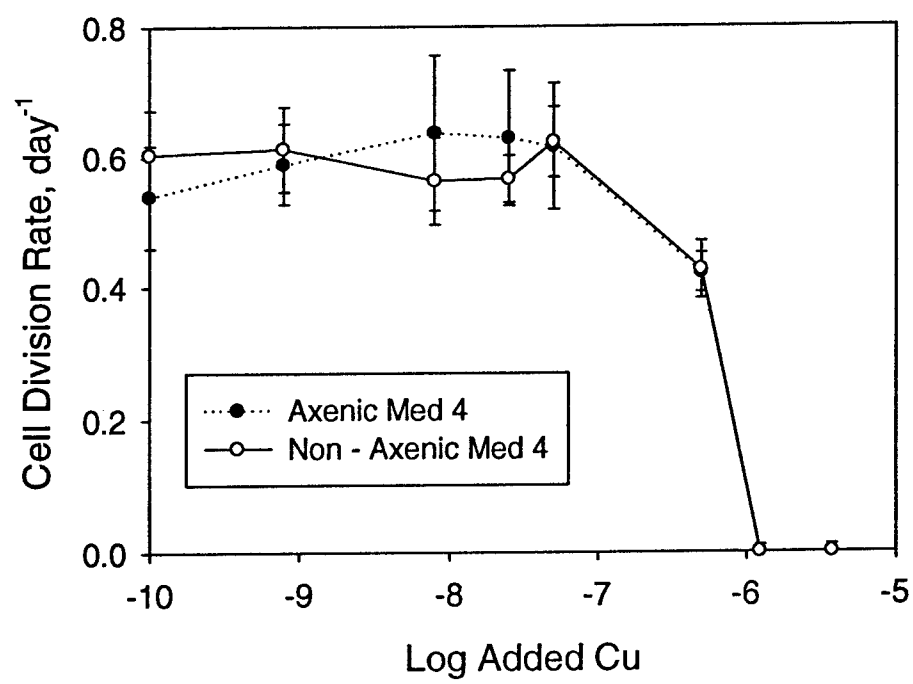| Media Component	Total concentration
Nitrilotriacetic acid (NTA)	10^{-4} M
NH_4Cl	50 μM
Urea	100 μM
NaH_2PO_4 H_2O	10 μM
Na_2CO_3	40 μM
$FeCl_3$ $6H_2O$	1.2 μM
Na_2MoO_4 2 H_2O	0.003 μM
$MnCl_2$ $4H_2O$	0.09 μM
$CoCl_2$ $6H_2O$	0.005 μM
$NiCl_2$ 6 H_2O	0.01 μM
Na_2SeO_3	0.01 μM
$ZnSO_4$ $7H_2O$	0.008 μM

Fig 2) Cell division rates as a function of free Cu^{2+} in axenic and non-axenic

Prochlorococcus cultures. The presence of bacteria in cultures of the

Prochlorococcus isolate Med 4 had no significant effect on the copper toxicity. The

cultures were grown in acid washed polycarbonate tubes in a 14:10 light dark cycle

at an irradiance of 100 $\mu Em^{-2}sec^{-1}$. The media composition is listed below, trace

metals were buffered with EDTA.

Composition of the EDTA trace metal media.

Media Component	Total concentration
EDTA	11.7 μM
NH_4Cl	50 μM
Urea	100 μM
$NaH_2PO_4\ H_2O$	10 μM
$FeCl_3\ 6H_2O$	1.2 μM
$Na_2MoO_4\ 2\ H_2O$	0.003 μM
$MnCl_2\ 4H_2O$	0.005 μM
$CoCl_2\ 6H_2O$	0.005 μM
$NiCl_2\ 6\ H_2O$	0.01 μM
Na_2SeO_3	0.01 μM
$ZnSO_4\ 7H_2O$	0.008 μM

Fig 3) On-deck bottle incubations: some details about methods. In order minimize

potential trace metal contamination from repeatedly sampling the on-deck bottle

incubations, samples were collected using positive pressure supplied by either

pushing air through the tubing with a syringe or a gas tank of a air and CO_2 mix. In

either case the air was filtered through two Acro disk filters before it reached the

incubation bottle. Samples were collected through the Teflon stop cock. All fittings

and tubing, with the exception of the flexible tubing connecting the Acro disk filters,

was Teflon. The incubation bottle itself was made of polycarbonate. All

components were acid cleaned and when possible were kept at a pH of 2 until

immediately before use when they were rinsed with clean seawater. The incubation

bottles were placed in bags made of several layers of optical gels to reduce the

incident irradiation to the desired level (see below). The Rosco series of gels is blue,

the other gels are neutral density filters. The outside layer was at least one heavy

duty transparent bag which was closed with a metal-free "Chip Clip".

Location	Depth Sampled	% Io	Optical gels used with their approximate percent transmissions
Northern Sargasso Well mixed	45m	20%	Rosco 69 (18% T)
Northern Sargasso Seasonally stratified	16m 85m	9% 2.3%	Rosco 69 (18% T) + light grey (50% T) Rosco 69 (18% T) + dark grey (12% T)
Southern Sargasso Permanently stratified	50m 125m	9% 2.3%	Rosco 69 (18% T) + light grey (50%T) Rosco 69 (18% T) + dark grey (12% T)

0.2μ Acro disk filters

1/4" ID flexible tubing

Tubing clamp

Teflon stop-cock

1/8" OD Teflon tubing

To pressure source

1/4" OD Teflon tubing

1/4" to 1/8" Teflon Reducing union

Teflon transfer cap

Polycarbonate bottle
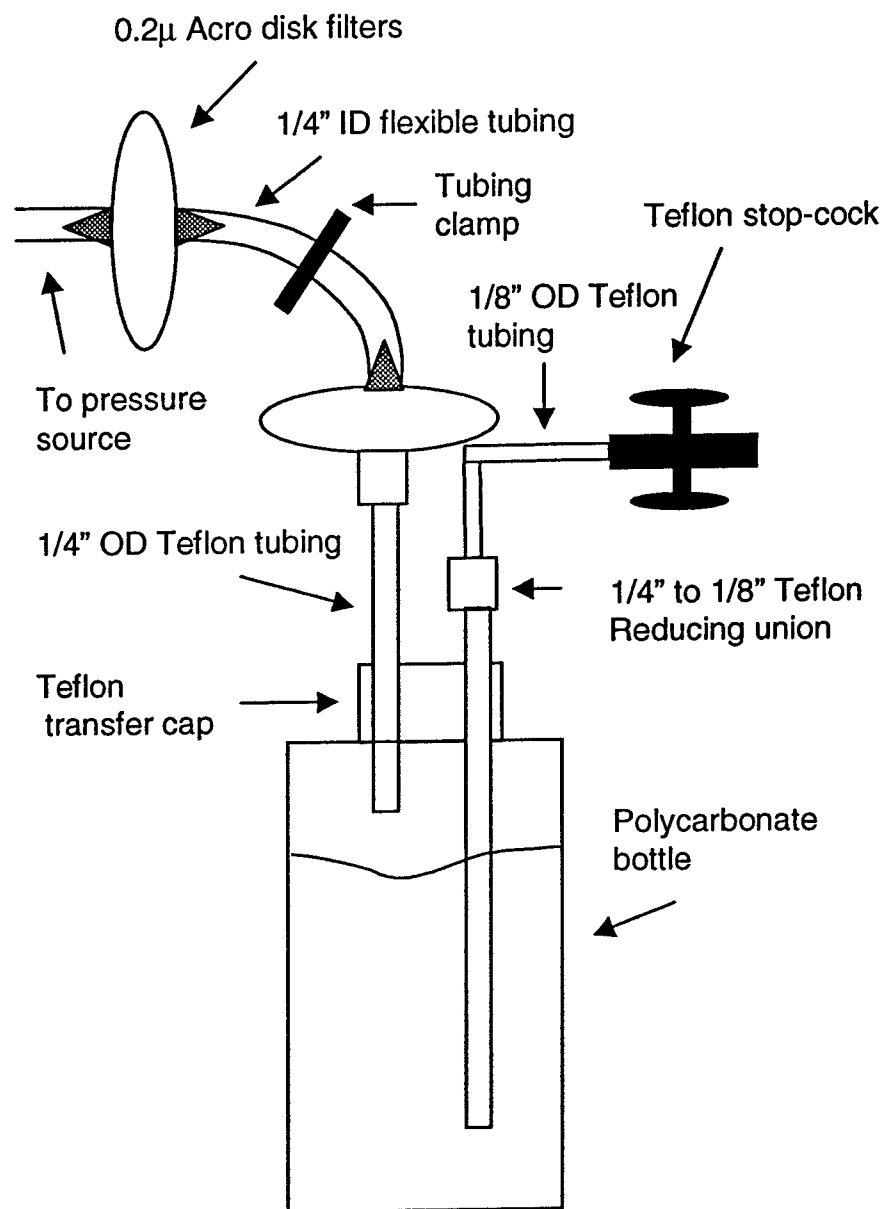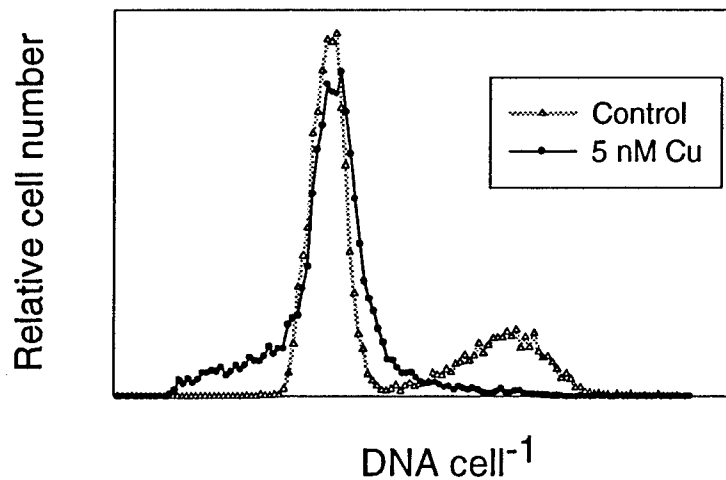
Fig 4) *Prochlorococcus* DNA histograms in the control and 5 nM Cu bottles from 125m in the permanently stratified water column 12 hours after the addition of copper. Samples were taken for DNA analysis at 20:00, at which time cells committed to division are expected to be in the S or G_2+M phases of the cell cycle. Note that the G_1 peak from the 5 nM Cu bottle has a left shoulder. DNA per cell may have been decreasing which is an indication that copper had a significant effect on the cell division cycle. Compare to Fig 8 in Chapter 3.
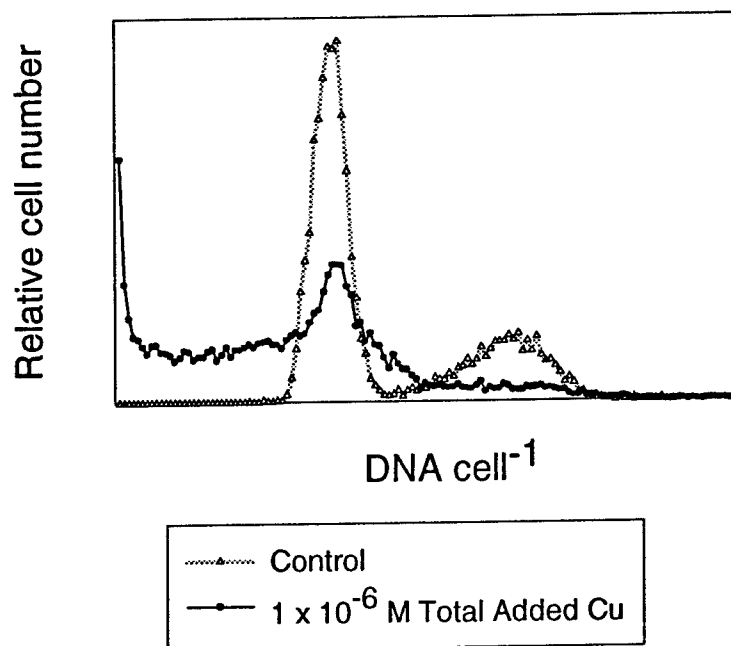
Fig 5) *Prochlorococcus* DNA histograms from axenic Med 4 cultures grown in constant light with 0 or 1.0×10^6 M total added copper. The media composition is the same as in Appendix B, Fig 2. Note that the G_1 peak from the + Cu sample has a left shoulder representing cells with degraded DNA. Cells in the + Cu sample appear to be arrested in the G_1 and early S phases of the cell cycle. Compare to Fig 8 in Chapter 3 and Appendix B, Fig 4.

Appendix C

Mortality rates in the Sargasso Sea.

INTRODUCTION

In chapter 4 cell division rates in the Sargasso Sea were determined in order to try to understand the mechanisms responsible for the observed patterns in cyanobacteria abundance. Mortality rates may also influence net growth rates and phytoplankton abundance. For instance, *Prochlorococcus* abundance could be low in shallow mixed layers in the northern Sargasso Sea because high mortality is coupled with low cell division rates. Picoplankton mortality can derive from a variety of sources including heterotrophic grazing (Landry et al. 1995b, Caron et al. 1999) and viral lysis. The importance of viral lysis in lowering cell numbers is unclear (Fuhrman and Suttle 1993, Waterbury and Valois 1993) but grazing is probably the predominant loss factor. In some environments trace metal toxicity may also be important (Chapter 3). Few *Prochlorococcus* are expected to be lost to sinking directly because of their small size (Pedros-Alio et al. 1989) although picoplankton can be incorporated into sinking particles through grazing activity. For instance, *Synechococcus* can be abundant in appendicularian fecal pellets (Gorsky et al. 1999) and organic aggregates (Waite et al. 2000). At depth, especially when the water column is well mixed, dilution with deep, essentially cell free water may also be an important sink for picoplankton.

The two methods used to calculate mortality rates in the Sargasso Sea in this study are based on changes in cell number. These mortality rates are considered to be only very rough estimates for two reasons. For one, the cell number data itself was in some cases highly variable. In the seasonally stratified water column in particular, the shoaling of

the thermocline simultaneously increased cell numbers in the mixed layer and decreased

them at depth (Chapter 4). The day to day variability in grazing rates seen in other

studies (Caron et al. 1999) was also significant. This issue could not be addressed in this

study due to the relatively short durations of the experiments (only one light dark cycle

was studied). With the inherent variability in cell number data and grazing rate data in

mind, it was possible to distinguish some general trends.

METHODS

Cell division and net growth rate method. For a glossary of important terms see the

following page. The mortality rate (m_{dna}, day^{-1}) was calculated from the net specific

growth rate (k, day^{-1}) and the cell division rate (μ_{dna}, day^{-1}) (Vaulot et al. 1995, Liu et al.

1997). The net growth rate was calculated as

$$k = \frac{\ln N_t - \ln N_{t_0}}{t - t_0} \quad (1)$$

Where N_t and N_{t_0} are the cell numbers at time t (the beginning of the day) and t_0 (one

day later). Because of the variability in the cell number data, the first two (in the

beginning of the day) and the last two data points were averaged to determine the t and t_0

values, respectively. μ_{dna} day^{-1} was calculated in Chapter 4.

Once values of μ_{dna} and k are on hand, the mortality rate can then be determined as

$$\mu_{dna} = k + m_{dna} \qquad (2)$$

Glossary of important terms

Parameter	Units	Definition
μ	day^{-1}	Cell division rate: the growth rate in the absence of cell losses.
μ_{dna}	day^{-1}	Calculated from cell cycle analysis using Eq. 1 from Chapter 4.
μ_{min}	day^{-1}	The minimum cell division rate, calculated from DNA histograms taken at one time point using Eq. 2 from Chapter 4.
k	day^{-1}	Net growth rate: $k = \mu - m$ day^{-1} Eq. 1 from Appendix C. Cell division rate minus cell mortality.
m	day^{-1}	Mortality: cell losses.
m_{dna}	day^{-1}	Calculated from the net growth rate and the μ_{dna} using Eq. 2 from Appendix C.
$m_{cell\#}$	day^{-1}	Calculated from the decline in cell numbers when cell division is not taking place.

Cell number method. *Prochlorococcus* mortality rates ($m_{cell\#}$), were also calculated from the slope of the decrease in abundance over the time interval when the cells were not dividing (Vaulot et al. 1995, Liu 1998). Data points used in this calculation had less then 5% of the cells in the S or G_2+M phases. This method assumes that mortality remains constant throughout the day, which may not be true. For instance, protozoan grazing rates in some studies are higher in the day than they are at night (Weisse 1989, Liu et al. 1995, Dolan and Simek 1999). In other cases the reverse occurs and mortality rates seem to be higher during the night when *Prochlorococcus* divide (Liu et al. 1997, Chapter 2).

RESULTS AND DISCUSSION

Mortality rates estimated using the cell division and net growth rates were generally lower then those estimated from the decline in cell numbers alone. This discrepancy is most likely due to variability in the cell number data, which are a function of both cell division and mortality. For instance, cell numbers were particularly variable in the seasonally stratified water column where the thermocline shoaled by approximately 10m during the second half of the night (Chapter 4). Depths where a 10m difference in depth would make a large difference in cell numbers, i.e. where there was a steep gradient in cell numbers, were the most affected. For this reason determining mortality rates using the net growth rate was not done in this water column except at 80m where the gradient in cell numbers were relatively shallow. Cell cycle parameters, which are intrinsic cell properties and more uniform with depth, are less likely to be seriously affected by changes in water column stability. $m_{cell\#}$ values were determined before the shoaling of

the thermocline in the seasonally stratified water column and were calculated for every depth except 100m. At this depth S phase initiation occurred earlier (Chapter 4) and as a result there were too few data points with a low percentage of cells in S or G_2+M to calculate $m_{cell\#}$ early in the day.

Prochlorococcus cell division and mortality rates were generally well balanced in the permanently stratified water column. At both 50 and 125m, m_{dna} rates were 0.5 day^{-1}. These are close to the cell division rates (μ_{dna} were 0.4 and 0.5 day^{-1}) at the same depths (Table 1). In the seasonally stratified water column cell division and mortality rates were also well balanced at 80m (Table 1). Balanced cell division and mortality is in agreement with earlier studies from the subtropical (Liu et al. 1997) and equatorial Pacific (Landry et al. 1995a, Vaulot et al. 1995, Verity et al. 1996, Liu et al. 1997, Mann and Chisholm in press, Chapter 2) and the Arabian Sea (Liu 1998).

In the seasonally stratified water column, mortality ($m_{cell\#}$) declined towards the surface from 0.6 day^{-1} at 80m to 0.2 day^{-1} at 15m (Table 1). These values were only calculated over a short time period, but the low mortality rate at the surface, where the *in situ* free Cu^{2+} is 3.6 pM (Chapter 3), is consistent with the fact that free Cu^{2+} greater than 1 pM can inhibit ciliate growth rates in culture (Stoecker et al. 1986). However, the variability found in other studies in both heterotroph concentrations and grazing rates with depth is high. In some cases the abundance of heterotrophic nanoflagellates and mixotrophic nanoplankton, both of which consume cyanobacteria, can be higher at 0 than 100m in the Sargasso Sea (Arenovski 1994, Caron et al. 1999). In other water columns heterotrophic

nanoflagellate concentrations did not vary significantly with depth (Weisse and Scheffel-Möser 1991, Arenovski 1994, Caron et al. 1999). Grazing rates measured using the disappearance of labeled prey in surface waters from the Sargasso Sea were also variable and ranged from undetectable to 0.6 day^{-1} (Caron et al. 1999). There was no clear difference between the grazing rate at the surface and at 100 to 120m in this study (Caron et al. 1999). Even if the concentration of heterotrophic flagellates is high in the surface waters this may not necessarily imply that grazing pressure on *Prochlorococcus* increased. For instance, there is a general correlation between nanoflagellate and bacteria abundance (Sanders et al. 1992), so nanoflagellates may have increased at the surface because the concentration of bacteria and *Synechococcus* increased as well (Arenovski 1994). In addition, marine protozoa in the laboratory are least likely to prefer *Prochlorococcus* as prey. Higher cell division rates and cell yields can be obtained from *Synechococcus* and preferably bacteria (Caron et al. 1991).

Prochlorococcus cell division and mortality rates in the winter tended to be unbalanced over the time period sampled. In the well mixed water column, deteriorating weather conditions prevented a full 24 hour sampling period. In this case the net growth rate was probably overestimated, which would lead to an underestimate of m_{dna} (Eq. 2). Even with the existing data, the m_{dna} at 45m of 0.3 day^{-1} was more than twice the cell division rates (Table 1). An imbalance between cell division and mortality and a negative growth rate is consistent with the decline of *Prochlorococcus* numbers during the winter. However, this degree of imbalance may not persist as it would take less than two weeks for the population to decline by an order of magnitude under these conditions.

Table 1) Summary of cell division and mortality rates, day^{-1}.

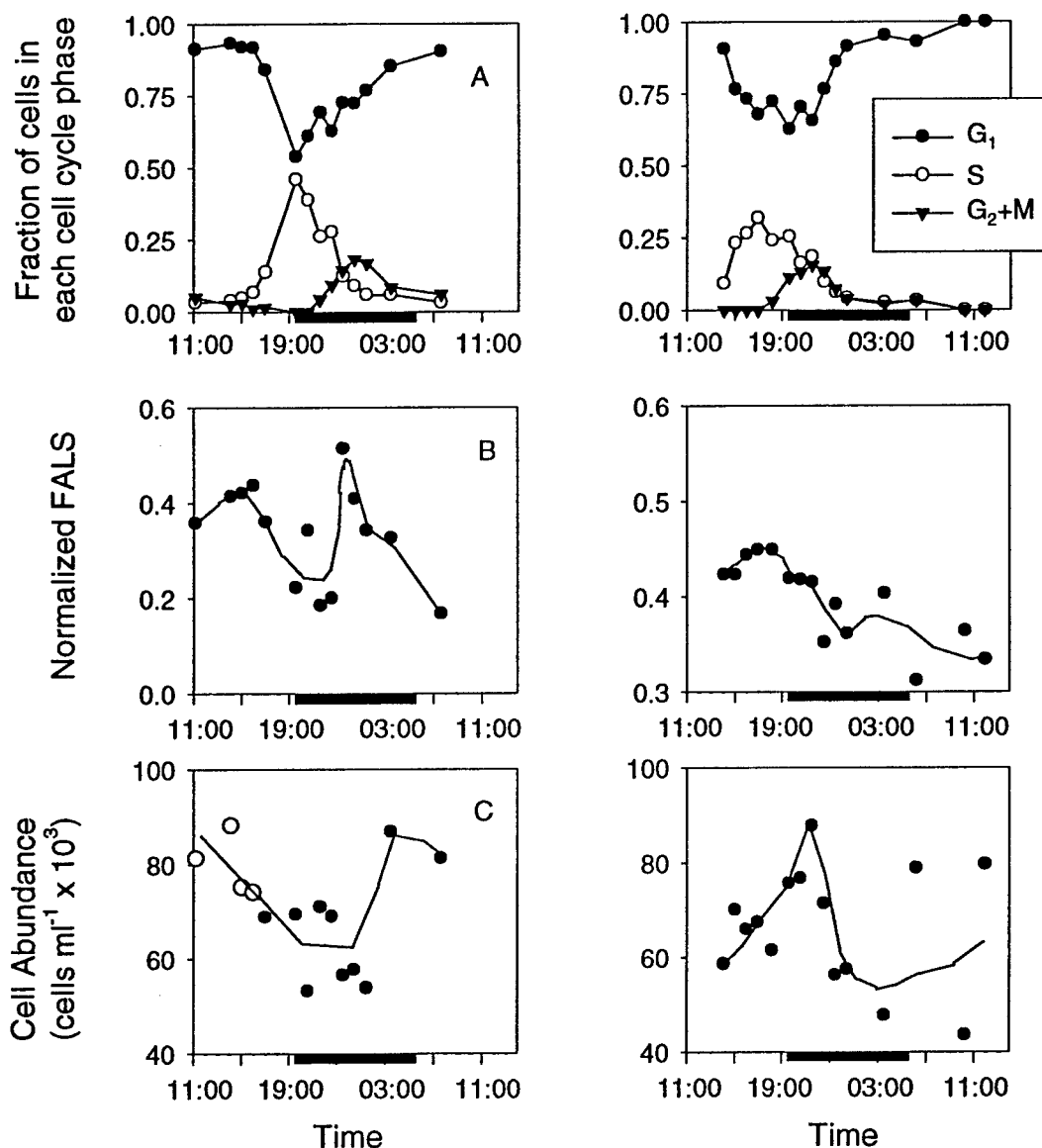| Station	Depth	μ_{min}	μ_{dna}	m_{dna}	$m_{cell\#}$	k
Northern	0	0.2				
Sargasso:	15	0.1				
well mixed	45	0.2	0.1	0.3	0.5	-0.2
	80	0.1				
	150	0.0				
Northern	15				0.2	
Sargasso:	30				0.5	
seasonally	80	0.4	0.4	0.4	0.6	0.0
stratified	100	0.3	0.3			
Southern	0	0.3				
Sargasso:	50	0.3	0.4	0.5	0.4	-0.1
permanently	80	0.4				
stratified	125	0.4	0.5	0.5	0.5	0.0
	150	0.2				

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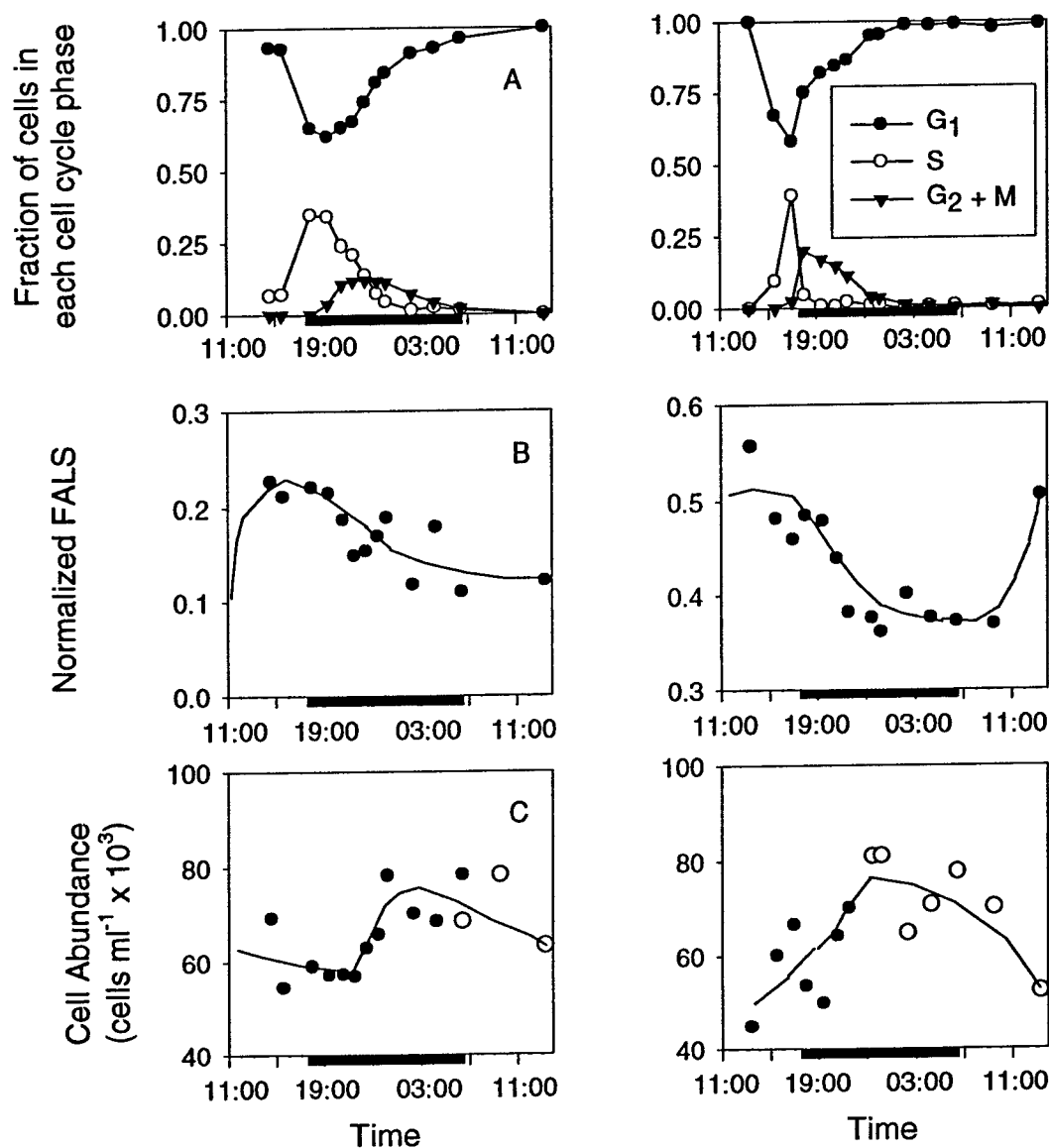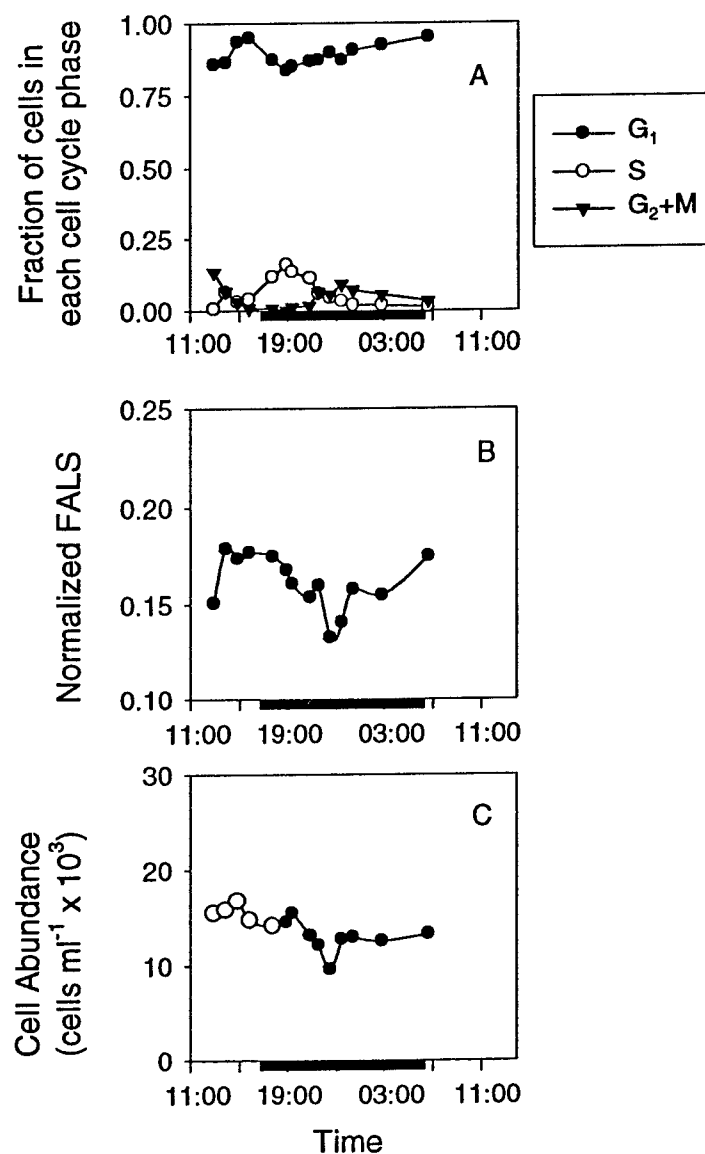
Fig 1) Diel patterns seen in *Prochlorococcus* for samples from 50m (left panel) and 125m (right panel) in a permanently stratified water column in the southern Sargasso Sea. The mixed layer depth was 75m. **A)** Fraction of cells in each cell cycle phase, **B)** FALS per cell normalized to standard beads and **C)** cell abundance over the light dark cycle. Data points in white were used to determine the mortality rate using the cell number method (see text). The solid bar represents the dark period.

Fig 2) Diel patterns seen in *Prochlorococcus* for samples from 80m (left panel) and 100m (right panel) in a seasonally stratified water column in the northern Sargasso Sea. The mixed layer depth was 25m. **A)** Fraction of cells in each cell cycle phase, **B)** FALS per cell normalized to standard beads and **C)** cell abundance over the light dark cycle. Data points in white were used to determine the mortality rate using the cell number method (see text). The solid bar represents the dark period.

Fig 3) Diel patterns seen in *Prochlorococcus* for samples from 45m in a well mixed
water column in the northern Sargasso Sea. The mixed layer depth was 200m.
A) Fraction of cells in each cell cycle phase, **B)** FALS per cell normalized to standard
beads and **C)** cell abundance over the light dark cycle. Data points in white were used
to determine the mortality rate using the cell number method (see text).
The solid bar represents the dark period.

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16. Abstract (Limit: 200 words)

The marine cyanobacteria *Synechococcus* and *Prochlorococcus* are important primary producers in oligotrophic oceans. The abundance and cell division rates of these cyanobacteria can be influenced by trace metals such as iron and copper. Iron is an essential trace metal that is present in the high nutrient, low chlorophyll waters of the equatorial Pacific in extremely low concentrations. When these waters were enriched with iron, *Prochlorococcus* cell division rates doubled indicating that these cells were iron limited. Mortality rates nearly doubled as well and as a result cell numbers remained constant. Copper is also essential for growth, but in the Sargasso Sea it can be present in toxic, rather than limiting concentrations. We hypothesize that ambient copper levels may influence the relative abundance and cell division rates of *Prochlorococcus* and *Synechococcus* in the Sargasso Sea. *Prochlorococcus* isolates were more sensitive to copper than *Synechococcus*, and in the field there was a rough inverse correlation between *Prochlorococcus* abundance and free Cu^{2+}. Net growth rates were substantially reduced when *Prochlorococcus* from environments where the *in situ* free Cu^{2+} was low were exposed to copper. The distribution of *Synechococcus* is more uniform and they were relatively copper resistant across a range of environments.

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